MUTANTS OF STREPTOCOCCAL TOXIN A AND METHODS OF USE

BACKGROUND OF THE INVENTION

Streptococcus pyogenes, also known as β-hemolytic group A streptococci (GAS) is a pathogen of humans which can cause mild infections such as pharyngitis

5 and impetigo. Post infection autoimmune complications can occur, namely rheumatic fever and acute glomerulonephritis. GAS also causes severe acute diseases such as scarlet fever and streptococcal toxic shock syndrome (STSS). Severe GAS infections were a large problem in the U.S. and throughout the world at the beginning of this century. In the mid-forties, the number of cases and their

10 severity decreased steadily for yet not completely understood reasons. However, more recently, a resurgence of serious diseases caused by GAS has been seen such that there may be 10-20,000 cases of STSS each year in the United States. As many as 50 to 60% of these patients will have necrotizing fascitis and myositis; 30 to 60% will die and as many as one-half of the survivors will have limbs amputated.

15 In 1986 and 1987 two reports described an outbreak of severe GAS infections localized in the Rocky Mountain area. These reports have been followed in the past few years by several others describing a disease with analogous clinical presentation. The symptoms described for this disease were very similar to those described for toxic shock syndrome (TSS), and in 1992 a committee of scientists

20 gave to this clinical presentation the formal name of STSS, and set the criteria for its diagnosis. STSS is defined by the presence of the following:

1.   hypotension and shock;

2.   isolation of group A streptococci;

3.   two or more of the following symptoms: fever 38.5°C or higher,

25 scarlet fever rash, vomiting and diarrhea, liver and renal dysfunction, adult respiratory distress syndrome, diffuse intravascular coagulation, necrotizing fascitis and/or myositis, bacteremia.

Streptococcal isolates from STSS patients are predominantly of M type 1 and 3, with M18 and nontypable organisms making up most of the reminder. The

30 majority of M1, 3, 18, and nontypable organisms associated with STSS make pyrogenic exotoxin A (SPE-A, scarlet fever toxin A). In contrast, only 15% of general streptococcal isolates produce this toxin. Moreover, administration of

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SPE-A to a rabbit animal model and in two accidental human inoculations can reproduce the symptoms of STSS.

SPE-A is a single peptide of molecular weight equal to 25,787 daltons, whose coding sequence is carried on the temperate bacteriophage T12. *speA*, the

5   gene for SPE-A, has been successfully cloned and expressed in Escherichia coli. SPE-A is a member of a large family of exotoxins produced by streptococci and staphylococci, referred to as pyrogenic toxins based upon their ability to induce fever and enhance host susceptibility up to 100,000 fold to endotoxin.

Recently these toxins have been referred to as superantigens because of their

10   ability to induce massive proliferation of T lymphocytes, regardless of their antigenic specificity, and in a fashion dependent on the composition of the variable part of the β chain of the T cell receptor. These toxins also stimulate massive release of IFN-γ, IL-1, TNF-α and TNF-β. Other members of this family are streptococcal pyrogenic exotoxins type B and C, staphylococcal toxic shock syndrome toxin 1,

15   staphylococcal enteroxtoxins A, B, Cn, D, E, G and H, and non-group A streptococcal pyrogenic exotoxins. These toxins have similar biochemical properties, biological activities and various degrees of sequence similarity.

The most severe manifestations of STSS are hypotension and shock, that lead to death. It is generally believed that leakage of fluid from the intravascular to the

20   interstitial space is the final cause of hypotension, supported by the observation that fluid replacement therapy is successful in preventing shock in the rabbit model of STSS described above. It has been hypothesized that SPE-A may act in several ways on the host to induce this pathology.

SPE-A has been shown to block liver clearance of endotoxin of endogenous

25   flora's origin, by compromising the activity of liver Kuppfer cells. This appears to cause a significant increase in circulating endotoxin, that through binding to lipopolysaccharide binding protein (LBP) and CD14 signaling leads to macrophage activation with subsequent release of TNF-α and other cytokines. Support for the role of endotoxin in the disease is given by the finding that the lethal effects of

30   SPE-A can be at least partially neutralized by the administration to animals of polymyxin B or by the use of pathogen free rabbits.

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Another modality of induction of shock could be the direct activity of the toxin on capillary endothelial cells. This hypothesis stems from the finding that the staphylococcal pyrogenic toxin TSST-1 binds directly to human umbilical cord vein cells and is cytotoxic to isolated porcine aortic endothelial cells.

5   Another of the toxin's modality of action includes its superantigenicity, in which the toxin interacts with and activates up to 50% of the host T lymphocytes. This massive T cell stimulation results in an abnormally high level of circulating cytokines TNF-β and IFN-(which have direct effects on macrophages to induce release of TNF-α and IL-1. These cytokines may also be induced directly from 10   macrophages by SPE-A through MHC class II binding and signalling in the absence of T cells. The elevated levels of TNF-α and -β cause several effects typically found in Gram negative induced shock, among which is damage to endothelial cells and capillary leak. However, the administration to SPE-A treated rabbits of cyclosporin A, which blocks upregulation of IL-2 and T cell proliferation, did not 15   protect the animals from shock, suggesting that additional mechanisms may be more important in causing capillary leak.

Thus, there is a need to localize sites on the SPE-A molecule responsible for different biological activities. There is a need to develop variants of SPE-A that have changes in biological activities such as toxicity and mitogenicity. There is a 20   need to develop compositions useful in vaccines to prevent or ameliorate streptococcal toxic shock syndrome. There is also a need to develop therapeutic agents useful in the treatment of streptococcal toxic shock syndrome and other diseases.

SUMMARY OF THE INVENTION

25   This invention includes mutant SPE-A toxins and fragments thereof, vaccines and pharmaceutical compositions and methods of using vaccines and pharmaceutical compositions.

Mutant SPE-A toxins have at least one amino acid change and are 30   substantially nonlethal as compared with a protein substantially corresponding to a wild type SPE-A toxin. For vaccine compositions, mutant toxins also stimulate a protective immune response to at least one biological activity of a wild type SPE-A

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toxin. Mutant toxins for vaccine compositions are optionally further selected to
have a decrease in enhancement of endotoxin shock and a decrease in T cell
mitogenicity when compared to the wild type SPE-A. An especially preferred
mutant for vaccine compositions is one that has a change at an amino acid equivalent

5　　to amino acid 20 of a wild type SPE-A toxin. For pharmaceutical compositions, it is
preferred that a mutant toxin is substantially nonlethal while maintaining T cell
mitogenicity comparable to a wild type SPE-A toxin.

The invention also includes fragments of a wild type *speA* toxin and mutants
of *speA* toxins. Fragments and peptides derived from wild type SPE-A are mutant

10　　SPE-A toxins. Fragments can include different domains or regions of the molecule
joined together. A fragment is substantially nonlethal when compared to a wild type
SPE-A toxin. For mutant toxins, a fragment has at least one amino acid change
compared to a wild type SPE-A amino acid sequence. Fragments are also useful in
vaccine and pharmaceutical compositions.

15　　The invention also includes expression cassettes, vectors and transformed
cells. An expression cassette comprises a DNA sequence encoding a mutant SPE-A
toxin or fragment thereof operably linked to a promoter functional in a host cell.
DNA cassettes are preferably inserted into a vector. Vectors include plasmids or
viruses. Vectors are useful to provide template DNA to generate DNA encoding a

20　　mutant SPE-A toxin. DNA cassettes and vectors are also useful in vaccine
compositions. Nucleic acids encoding a mutant SPE-A toxin or fragment thereof
can be delivered directly for expression in mammalian cells. The promoter is
preferably a promoter functional in a mammalian cell. Nucleic acids delivered
directly to cells can provide for expression of the mutant SPE-A toxin in an

25　　individual so that a protective immune response can be generated to at least one
biological activity of a wild type SPE-A toxin.

Additional vaccine compositions include stably transformed cells or viral
vectors including an expression cassette encoding a mutant SPE-A toxin or fragment
thereof. Viral vectors such as vaccinia can be used to immunize humans to generate

30　　a protective immune response against at least one biological activity of a wild type
SPE-A toxin. Transformed cells are preferably microorganisms such as S. aureus, E.
coli, or Salmonella species spp. Transformed microorganisms either include mutant

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SPE-A toxin or fragment thereof on their surface or are capable of secreting the mutant toxin. Transformed microorganisms can be administered as live, attenuated or heat killed vaccines.

The invention also includes methods of using vaccines and pharmaceutical compositions. Vaccines are administered to an animal in an amount effective to generate a protective immune response to at least one biological activity of a wild type SPE-A toxin. Preferably, the vaccine compositions are administered to humans and protect against the development of STSS. Pharmaceutical compositions are used in methods of stimulating T cell proliferation. The pharmaceutical compositions are especially useful in the treatment of cancers that are treated with interleukins, interferons or other immunomodulators, T cell lymphomas, ovarian and uterine cancers. A pharmaceutical composition is administered to a patient having cancer.

The mutant SPE-A toxins and/or fragments thereof and other vaccine compositions can be useful to generate a passive immune serum. Mutant SPE-A toxins or fragments thereof, DNA expression cassettes or vectors, or transformed microorganisms can be used to immunize an animal to produce neutralizing antibodies to at least one biological activity of wild type SPE-A. The neutralizing antibodies immunoreact with a mutant SPE-A toxin and/or fragment thereof and the wild type SPE-A toxin. Passive immune serum can be administered to an animal with symptoms of A streptococcal infection and STSS.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 Ribbon drawing of the modeled 3-dimensional structure of streptococcal pyrogenic exotoxin A. Domain A and B are indicated.

Figure 2 View of SPE-A as seen from the back in reference to the standard view seen in Figure 1. Numbered residues are those homologous to residues in TSST-1 evaluated for reduced systemic lethality.

Figure 3 shows the DNA sequence (SEQ ID NO:12) and predicted amino acid sequence (SEQ ID NO:13) of the cloned SPE-A toxin from T12.

Figure 4 T cell proliferation assay. Rabbit splenocytes were incubated in 96 well microtiter plates in quadruplicate with SPE-A, K16N-SPE-A, and N20D-

SPE-A for 72 hours. Cells were pulsed with [3H] thymidine for 18 to 24 hours,

harvested onto filters, and [3H] thymidine incorporation was measured in a

scintillation counter. Results are expressed as counts per minute (CPM) versus

concentrations of toxin in μg/ml. Data presented are from the most representative of

5   three independent experiments.

Figure 5 T cell proliferation assay. Rabbit splenocytes were incubated in 96

well microtiter plates in quadruplicate with SPE-A, C87S-SPE-A, C98S-SPE-A, and

C90S-SPE-A for 72 hours. Cells were pulsed with [3H] thymidine for 18 to 24

hours, harvested onto filters, and [3H] thymidine incorporation was measured in a

10   scintillation counter. Results are expressed as counts per minute (CPM) versus

concentrations of toxin in μg/ml. Data presented are from the most representative of

three independent experiments.

Figure 6 T cell proliferation assay. Rabbit splenocytes were incubated in 96

well microtiter plates in quadruplicate with SPE-A, K157E-SPE-A, and S195A-

15   SPE-A for 72 hours. Cells were pulsed with [3H] thymidine for 18 to 24 hours,

harvested onto filters, and [3H] thymidine incorporation was measured in a

scintillation counter. Results are expressed as counts per minute (CPM) versus

concentrations of toxin in μg/ml. Data presented are from the more representative of

three independent experiments.

20   Figure 7. Superantigenicity of wild type SPE A compared to single mutant.

Rabbit spleen cells were incubated for 4 days with SPE A or mutants at the indicated

doses. Four replicate wells were used at each dose of SPE A and mutants. On day

3, 1 μCI 3H thymidine was added to each well. Superantigenicity index = 3H

thymidine incorporation by splenocytes in the presence of SPE A or mutants divided

25   by 3H thymidine incorporation in the absence of SPE A or mutants.

Figure 8. Superantigenicity of wild type SPE A compared to double mutants.

Methods used were those described in Figure 7.

Figure 9. SPE A Inhibition by Immunized Rabbit Sera. Rabbit sera from

rabbits immunized with single and double mutants was used to demonstrate the

30   ability of the sera to neutralize splenocyte mitogenicity in the presence of SPE A.

Figure 10 shows a front view of a ribbon structure of SPE-A oriented to

show locations contacting major histocompatibility complex type II in a complex.

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Figure 11 shows a front view of a ribbon diagram of SPE-A oriented to show locations that contact the T cell receptor in a complex.

Figure 12 shows a rear view of a ribbon structure of SPE-A oriented to show residues of the central α helix that form the floor of the groove that contacts the liver renal tubular cell receptor in a complex with this receptor.

Figure 13 shows a front (standard) view of a ribbon diagram of the modeled 3-dimensional structure of SPE A. Organized structures such as β-strands and α-helices are represented. Domains A and B are indicated. Alpha carbons of the mutated residues and certain other residues are represented as spheres.

DETAILED DESCRIPTION OF THE INVENTION

This invention is directed to mutant SPE-A toxins and fragments thereof, vaccine and pharmaceutical compositions including mutant SPE A toxins or fragments thereof, methods of preparing mutant SPE-A toxins and fragments thereof, and methods of using SPE-A toxins and fragments thereof.

Mutant SPE-A toxins are proteins that have at least one amino acid change and have at least one change in a biological function compared with a protein substantially corresponding to a wild type SPE-A toxin. Preferably, the mutant SPE-A toxin is substantially nonlethal when compared to a wild type SPE-A toxin at the same dose. Mutant SPE-A toxins can be generated using a variety of methods including site-directed mutagenesis, random mutagenesis, conventional mutagenesis, in vitro mutagenesis, spontaneous mutagenesis and chemical synthesis. Mutant SPE-A toxins are preferably selected to: 1) ensure at least one change in an amino acid; and 2) to have a change in at least one biological function of the molecule preferably a decrease or elimination of systemic lethality. The mutant toxins are useful in vaccine compositions for protection against at least one biological activity of SPE-A toxin such as prevention or amelioration of STSS, in methods of treating animals with symptoms of STSS, and in methods for stimulating T cell proliferation and in the treatment of cancer. Single, double, and triple SPE-A mutants were tested and the antibody to the mutants inhibited cell responses to SPE A.

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A.   **Mutant SPE-A Toxins or Fragments Thereof, Vaccine and
     Pharmaceutical Compositions**

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The invention includes mutant SPE-A toxins that have at least one amino
acid change and that have at least one change in a biological activity compared with
proteins that substantially correspond to and have the same biological activities as
wild type SPE-A.

Wild type SPE-A toxin is encoded by a gene *speA* found on bacteriophage
T12. The wild type SPE-A toxin has a molecular weight of 25,787 Daltons as

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calculated from the deduced amino acid sequence of the mature protein. A DNA
sequence encoding a wild type SPE-A toxin and the predicted amino acid sequence
for a wild type SPE-A toxin is shown in Figure 3. A DNA sequence encoding a wild
type SPE-A toxin has been cloned in E. coli and S. aureus. Amino acid number
designations in this application are made by reference to the sequence of Figure 3

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with glutamine at position 31 designated as the first amino acid. The first 30 amino
acids represent a leader sequence not present in the mature protein.

A structural model of a wild type SPE-A toxin is shown in Figure 1. The
structural model was constructed by homology modeling using Insight/Homology
program available from BioSym Corp., San Diego, CA. The model indicates that

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the wild type SPE-A toxin has several distinct structural features. These structural
features include: helix 2 (amino acids 11-15); N-terminal alpha helix 3 (amino acids
18-26); helix 4 (amino acids 64-72); central-α helix 5 (amino acids 142-158); helix 6
(amino acids 193-202); Domain B beta strands including strand 1 (amino acids 30-
36), strand 2 (amino acids 44-52), strand 3 (amino acids 55-62), strand 4 (amino

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acids 75-83), strand 5 (amino acids 95-106); Domain A beta strands including strand
6 (amino acids 117-126), strand 7 (amino acids 129-135), strand 8 (amino acids 169-
175), strand 9 (amino acids 180-186), and strand 10 (amino acids 213-220). In
addition, cysteine residues at residues 87, 90, and 98 may be important in formation
of putative disulfide bonds or maintaining local 3-D conformation.

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The wild type SPE-A toxin has several biological activities. These
biological activities include: 1) fever; 2) STSS; 3) systemic lethality due to
development of STSS or enhancement of endotoxin shock; 4) enhancing endotoxin
shock; 5) induction of capillary leak and hypotension; 6) inducing release of

cytokines such as IFN g, IL-1, TNF-α and TNF-β; 7) binding to porcine aortic endothelial cells; 8) binding to MHC class II molecules; 9) binding to T-cell receptors; and 10) T-cell mitogenicity (superantigenicity). These activities can be assayed and characterized by methods known to those of skill in the art.

5   As used herein, the definition of a wild type SPE-A toxin includes variants of a wild type SPE-A toxin that have the same biological activity of wild type SPE-A toxin. These SPE-A toxins may have a different amino acid or their genes may have a different nucleotide sequence from that shown in Figure 3 but do not have different biological activities. Changes in amino acid sequence are phenotypically silent.

10   Preferably, these toxin molecules have systemic lethality and enhance endotoxin shock to the same degree as wild type SPE-A toxin shown in Figure 3. Preferably these toxins have at least 60-99% homology with wild type SPE-A toxin amino acid sequence as shown in Figure 3 as determined using the SS2 Alignment Algorithm as described by Altschul,S. F., Bull. Math. Bio. 48:603 (1986). Proteins that have

15   these characteristics substantially correspond to a wild type SPE A.

          A mutant SPE-A toxin is a toxin that has at least one change in a amino acid compared with a protein substantially corresponding to a wild type SPE-A toxin. The change can be an amino acid substitution, deletion, or addition. There can be more than one change in the amino acid sequence, preferably 1 to 6 changes. It is

20   preferred that there are more than one change in the amino acid sequence to minimize the reversion of mutant SPE-A toxin to the wild type SPE-A toxin having systemic lethality or toxicity. For mutant SPE-A toxins useful in vaccines, it is preferred that the change in the amino acid sequence of the toxin does not result in a change of the toxin's ability to stimulate an antibody response that can neutralize

25   wild type SPE-A toxin. For mutant SPE-A toxins useful in vaccines, it is especially preferred that the mutant toxins are recognized by polyclonal neutralizing antibodies to SPE-A toxin such as from Toxin Technologies in Boca Raton, Fla. or Dr. Schlievert (University of Minnesota, Minneapolis, MN) and that the proteolytic profile is not altered compared with wild type SPE-A.

30   The changes in the amino acid sequence can be site-specific changes at one or more selected amino acid residues of a wild type SPE-A toxin. Site-specific changes are selected by identifying residues in particular domains of the molecule as

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described previously or at locations where cysteine residues are located. Site-specific changes at a particular location can optionally be further selected by determining whether an amino acid at a location or within a domain is identical to or has similar properties to an equivalent residue in other homologous molecules by

5   comparison of primary sequence homology or 3-D conformation. A homologous molecule is one that can be identified by comparison of primary sequence homology using the SS2 alignment algorithm of Altschul et al., cited supra or a homology modeling program such as Insight/Homology from BioSym, San Diego, CA. A homologous molecule is one that displays a significant number, typically 30-99%, of

10   identical or conservatively changed amino acids or has a similar three dimensional structure, typically RMS error for conserved regions of <2 Angstroms, when compared to a wild type SPE-A toxin.

Changes in the amino acid sequence at a particular site can be randomly made or specific changes can be selected. Once a specific site is selected it is

15   referred to by its amino acid number designation and by the amino acid found at that site in the wild type SPE-A as shown in Figure 3. The amino acid number designations made in this application are by reference to the sequence in Figure 3 with the glutamine at position 31 being counted as the first amino acid. Equivalent amino acids corresponding to those identified at a particular site in proteins

20   substantially corresponding to a wild type SPE-A toxin may have different amino acid numbers depending on the reference sequence or if they are fragments. Equivalent residues are also those found in homologous molecules that can be identified as equivalent to amino acids in proteins substantially corresponding to a wild type SPE-A toxin either by comparison of primary amino acid structure or by

25   comparison to a modeled structure as shown in Figure 1 or by comparison to a known crystal structure of a homologous molecule. It is intended that the invention cover changes to equivalent amino acids at the same or similar locations regardless of their amino acid number designation.

If a specific substitution is selected for an amino acid at a specific site, the

30   amino acid to be substituted at that location is selected to include a structural change that can affect biological activity compared with the amino acid at that location in the wild type SPE-A. The substitution may be conservative or nonconservative.

Substitutions that result in a structural change that can affect biological activity include: 1) change from one type of charge to another; 2) change from charge to noncharged; 3) change in cysteine residues and formation of disulfide bonds; 4) change from hydrophobic to hydrophilic residues or hydrophilic to hydrophobic residues; 5) change in size of the amino acids; 6) change to a conformationally restrictive amino acid or analog; and 7) change to a non-naturally occurring amino acid or analog. The specific substitution selected may also depend on the location of the site selected. For example, it is preferred that amino acids in the N-terminal alpha helix have hydroxyl groups to interact with exposed amide nitrogens or that they be negatively charged to interact with the partial positive charge present at the N-terminus of the α helix.

Mutant toxins may also include random mutations targeted to a specific site or sites. Once a site is selected, mutants can be generated having each of the other 19 amino acids substituted at that site using methods such as described by Aiyar et al., Biotechniques 14:366 (1993) or Ho et al. Gene 77:51-54 (1984). In vitro mutagenesis can also be utilized to substitute each one of the other 19 amino acids or non-naturally occurring amino acids or analogs at a particular location using a method such as described by Anthony-Cahill et al., Trends Biochem. Sci. 14:400 (1989).

Mutant toxins also include toxins that have changes at one or more sites of the molecule not specifically selected and that have a change in amino acids that is also not specifically selected but can be any one of the other 19 amino acids or a non-naturally occurring amino acid.

Substitutions at a specific site can also include but are not limited to substitutions with non-naturally occurring amino acids such as 3-hydroxyproline, 4-hydroxyproline, homocysteine, 2-aminoadipic acid, 2-aminopimilic acid, ornithine, homoarginine, N-methyllysine, dimethyl lysine, trimethyl lysine, 2,3-diaminopropionic acid, 2,4-diaminobutryic acid, hydroxylysine, substituted phenylalanine, norleucine, norvaline, g-valine and halogenated tyrosines. Substitutions at a specific site can also include the use of analogs which use non-peptide chemistry including but not limited to ester, ether and phosphoryl and boron linkages.

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The mutant toxins can be generated using a variety of methods. Those
methods include site-specific mutagenesis, mutagenesis methods using chemicals
such as EMS, or sodium bisulfite or UV irradiation, by spontaneous mutation, by in
vitro mutagenesis and chemical synthesis. Methods of mutagenesis can be found in

5   Sambrook et al., A Guide to Molecular Cloning, Cold Spring Harvard, New York
(1989). The especially preferred method for site-specific mutagenesis is using
asymmetric PCR with three primers as described by Perrin and Gilliland, 1990.
Nucleic Acid Res. 18:7433.

Once a mutant SPE-A toxin is generated having at least one amino acid

10   change compared with a protein substantially corresponding to the wild type SPE-A
toxin, the mutant SPE-A toxin is screened for nonlethality. It is preferred that
mutant SPE-A toxins selected from this screening are substantially nonlethal in
rabbits when administered using a miniosmotic pump (as described in Example 2) at
the same dose or a greater dose than a wild type SPE-A toxin. A mutant SPE-A

15   toxin or fragment thereof is substantially nonlethal if when administered to a rabbit
at the same dose as the wild type toxin less than about 10-20% of rabbits die.
Nonlethal mutant toxins are useful in vaccine and pharmaceutical compositions.
While not meant to limit the invention, it is believed that some amino acid residues
or domains that affect systemic lethality are separable from other biological

20   activities especially T cell mitogenicity.

For mutant toxins useful in vaccine composition it is further preferred that
the mutant SPE-A toxins are screened for those that can stimulate an antibody
response that neutralizes wild type SPE-A toxin activity. A method for selecting
mutant toxins that can stimulate an antibody response that neutralizes wild type

25   SPE-A toxin activity is to determine whether the mutant toxin immunoreacts with
polyclonal neutralizing antibodies to wild type SPE-A such as available from Toxin
Technologies, Boca Raton, Fla. or Dr. Schlievert. Methods of determining whether
mutant SPE-A toxins immunoreact with antibodies to wild type SPE-A toxin include
ELISA, Western Blot, Double Immunodiffusion Assay and the like.

30   Optionally, the mutant toxins can also be screened to determine if the
proteolytic profile of the mutant toxin is the same as the wild type SPE-A toxin. In
some cases, it is preferred that the mutants generated do not substantially change the

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overall three-dimensional conformation of the mutant toxin compared with the wild
type SPE-A toxin. One way of examining whether there has been a change in
overall conformation is to look at immunoreactivity of antibodies to wild type SPE-
A toxin and/or to examine the proteolytic profile of mutant SPE-A toxins. The

5   proteolytic profile can be determined using such enzymes as trypsin, chymotrypsin,
papain, pepsin, subtilisin and V8 protease in methods known to those of skill in the
art. The proteolytic profile of wild type SPE-A with the sequence shown in Figure 3
is known. The mutants that have a similar profile to that of wild type SPE-A are
selected.

10   Optionally, mutant toxins can also be screened and selected to have other
changes in biological activity. As described previously, there are several biological
activities associated with wild type SPE-A toxin. Those biological activities
include: 1) fever; 2) STSS; 4) enhancement of endotoxin shock; 5) capillary leak
and hypotension; 6) inducing release of cytokines such as IFN gamma, IL-1, TNF-α

15   and TNF-β; 7) binding to endothelial cells; 8) binding to MHC class II molecules; 9)
binding to T-cell receptors; and 10) T-cell mitogenicity (superantigenicity). These
biological activities can be measured using methods known to those of skill in the
art.

        For mutant SPE-A toxins or fragments thereof useful in vaccine

20   compositions, it is preferred that they are substantially nontoxic and immunoreactive
with neutralizing antibodies to wild type SPE-A . Neutralizing antibodies include
those that inhibit the lethality of the wild type toxin when tested in animals.
Optionally, mutant SPE-A toxins can have a change in one or more other biological
activities of wild type SPE-A toxin as described previously.

25   Optionally, preferred mutant toxins for vaccine compositions are further
screened and selected for a lack of potentiation of endotoxin shock. The preferred
assay for examining a lack of enhancement of endotoxin shock is described in
Example 4. Rabbits preferably have no demonstrable bacterial or viral infection
before testing. A lack of potentiation of or substantially no enhancement of

30   endotoxin shock is seen when less than about 25% of the animals develop shock
when the mutant SPE toxin is coadministered with endotoxin as compared to wild

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type SPE-A activity at the same dose. More preferably, none of the animals develop
shock.

Optionally, preferred mutants for vaccine compositions also are further
screened and selected for a change in T cell mitogenicity. A change in T-cell

5   mitogenicity can be detected by measuring T-cell proliferation in a standard 3H
thymidine assay using rabbit lymphocytes as described in Example 4; by measuring
levels of production of cytokines such as IFN gamma or TNF-β; by determining the
Vβ type of T cell response or by determining the interaction of the molecules with
MHC class II receptors. The preferred method for detecting a decrease in T-cell

10   mitogenicity is to measure T-cell proliferation of rabbit lymphocytes in the presence
and absence of the mutant toxin. Responses of T cells to wild type SPE-A toxin is
greatly enhanced above a normal in vitro response to an antigen. A substantial
decrease in T cell mitogenicity is seen when the mutant SPE-A toxin does not
stimulate a T cell proliferative response greater than the stimulation with an antigen

15   or negative control. Preferably, a decrease is seen such that the T cell proliferation
response to the mutant SPE-A toxin is no more than two-fold above background
when measured using rabbit lymphocytes at the same dose as the wild type SPE-A
toxin.

Optionally, the mutant SPE-A toxins useful in vaccine compositions are

20   further screened and selected for a decrease in capillary leak in endothelial cells.
The preferred method is using porcine aortic endothelial cells as described by Lee t
el., J. Infect. Dis. 164:711 (1991). A decrease in capillary leak in the presence of
mutant SPE-A toxins can be determined by measuring a decrease in release of a
radioactively labeled compound or by a change in the transport of a radioactively

25   labeled compound. A decrease in capillary leak is seen when the release or transport
of a radioactively labeled compound is decreased to less than about two fold above
background when compared with the activity of a wild type toxin.

The especially preferred mutant SPE-A toxins useful in vaccine
compositions are not biologically active compared with proteins that have wild type

30   SPE-A toxin activity. By nonbiologically active, it is meant that the mutant toxin
has little or no systemic lethality, has little or no enhancement of endotoxin shock
and little or no T cell mitogenicity. Preferably, the mutant SPE-A toxins selected for

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vaccine compositions substantially lack these biological activities, i.e., they react like a negative control or they stimulate a reaction no more than two-fold above background.

Changes in other biological activities can be detected as follows. Binding to
5   MHC class II molecules can be detected using such methods as described by
Jardetzky, Nature 368:711 (1994). Changes in fever can be detected by monitoring
temperatures over time after administration of the mutant SPE-A toxins. Changes in
the levels of cytokine production in the presence of mutant SPE-A toxins can be
measured using methods such as are commercially available and are described by
10   current protocols in immunology. (Ed. Coligan, Kruisbeck, Margulies, Shevach, and
Stroker. National Institutes of Health, John Wiley and Sons, Inc.)

Specific examples of mutant SPE-A toxins that have at least one amino acid
change and that are substantially nontoxic are described.

The especially preferred mutants for vaccine compositions are mutant SPE-A
15   toxins that immunoreact with polyclonal neutralizing antibodies to wild type SPE-A
toxin, are nontoxic, and optionally have a decrease in potentiation of endotoxin
shock and a decrease in T-cell mitogenicity. The especially preferred mutants have a
change in the asparagine at amino acid 20 such as the mutant N20D that has an
aspartic acid substituted for asparagine at residue 20 in the mature toxin (N20D).
20   The N20D mutant has been shown to be nontoxic, to have no enhancement of
endotoxin shock and a 5-fold decrease in T cell mitogenicity. In addition, changes at
amino acid 98 that result in a lack of a cysteine group at that location also result in a
mutant toxin that has a decrease in enhancement in endotoxin shock and a four-fold
decrease in mitogenicity. The especially preferred mutants at this location have a
25   serine substituted for a cysteine (C98S).

The preferred mutants for stimulation of T-cell proliferation and in the
treatment of cancer are those mutant toxins that are substantially nonlethal. It is
preferred that these mutant toxins retain T-cell mitogenicity at least at the level of
wild type SPE-A toxin. The especially preferred mutants have an amino acid change
30   at residue 157 of the wild type SPE-A such as substitution of glutamic acid for
lysine at that residue (K157E). The K157E mutant has been shown to be nonlethal
but retains mitogenicity comparable to the wild type SPE-A toxin.

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Mutants can be generated to affect a functional change by changing amino acids in a particular domain of a molecule as follows. A molecular model of wild type SPE-A toxin is shown in Figure 1. The especially preferred domains include the N-terminal α helix 3 (amino acids 18-26), the central α helix 5 (amino acids

5   142-158), the Domain B beta strands (amino acids 30-36; 44-52; 55-62; 75-83; and 95-106), and the Domain A beta strands (amino acids 117-126; 129-135; 169-175; 180-186; and 213-220). Cysteine residues at positions 87, 90, and 98 may also be important.

While not meant to limit the invention, it is believed that these domains form

10 specific 3-D conformations that are important in the biological functions of the wild type SPE-A activity. As can be seen in Figure 2, the N-terminal α helix and central α helix are closely situated so that residues here may be especially important in the toxicity of wild type SPE-A molecules. In addition , amino acids in the bordering B strands that are in close proximity to the central alpha helix may also be important in

15 toxicity. The molecular models as shown in Figures 1 and 2 help to identify surface residues and buried residues of the structural domains.

For vaccine compositions, changes are preferably made to the residues in N-terminal alpha helix 3 (residues 18-26) are screened and selected to decrease systemic lethality or enhancement of endotoxin or T cell mitogenicity or all three.

20 A specific example of a change in the N-terminal alpha helix 3 is a change in amino acid at residue 20. A change at this residue from asparagine to aspartic acid results in a decrease in enhancement of endotoxin shock, a decrease in systemic lethality, and a five-fold decrease in mitogenicity. Other changes at residue 20 are preferably those that change the distribution of charge at the surface residues or that

25 change the interaction of the N-terminal α helix with the central α helix. Substitutions at amino acid 20 with charged amino acids such as glutamic acid, lysine, arginine are likely to have the same effect. Changes made in this region are preferably those that decrease in systemic lethality due to STSS.

Preferably, changes are also made in the central α helix 5 residues 142-158.

30 Mutants in this region having at least one amino acid change are preferably selected for a decrease systemic lethality due to STSS. A similar central a helix identified in other toxin molecules has been shown to be associated with toxicity. A specific

17

example is a change at residue 157. Change at this residue from lysine to glutamic

acid results in a decrease in enhancement of endotoxin shock and systemic lethality

due to STSS.

However, T-cell mitogenicity is not affected by a change at this residue.

5   These results show that toxicity and enhancement of endotoxin shock are separable

activities from T cell mitogenicity. For vaccine compositions, other mutant toxins

with changes in this domain are optionally screened and selected for a decrease in T

cell mitogenicity. A change in the type of charge present at amino acid 157

indicates that a substitution of aspartic acid for the lysine is likely to have a similar

10   effect.

Preferably changes in domain B beta strands including residues 30-36 (beta

strand 1), residues 44-52 (beta strand 2), residues 55-62 (beta strand 3), residues 75-

83 (beta strand 4), and residues 95-106 (beta strand 5) (domain 5) are screened and

selected for nonlethality, and optionally for a decrease in enhancement of endotoxin

15   shock and/or T cell mitogenicity. Multiple residues that form N-terminal barrel of

beta sheet in several toxins such as SEB, SEA, TSST-1 have been shown to be

important for binding to MHC class II molecules. A decrease in MHC class II

binding by mutant toxins can also be selected by using assays such as described by

Jardetzky et al., cited supra. Changes to these residues that would disrupt beta sheet

20   conformation or change the contact residues with MHC class II molecules,

especially those on the concave surface of the beta barrel, are selected. See Figure 1.

For vaccine compositions, it is preferred that changes that may change local

conformation do not change the immunoreactivity of the mutant toxins with

polyclonal neutralizing antibodies to the wild type SPE-A toxin.

25   Preferably changes to Domain A beta strands, including residues 117-126

(domain beta strand 6), residues 129-135 (domain 7), residues 169-175 (domain 8),

residues 180-186 (domain 9), and residues 213-220 (domain 10), are selected to be

nonlethal, have a decrease in endotoxin shock, and/or have a decrease in T cell

mitogenicity. Changes that would alter the beta sheet conformation without

30   changing the immunoreactivity of the mutant SPE-A toxin with polyclonal

neutralizing antibodies to wild type SPE-A toxin are preferably selected.

18

Superpositioning the three-dimensional structures of four staphylococcal

superantigens (TSST-1, SEA, SEB, and SEC-3) and of SPE-A demonstrated that

these proteins share 18 structurally conserved amino acids (Table A). Using these

18 structurally conserved amino acid residues as reference points allows

5   superpositioning of the structures of these 5 proteins with RMS differences at or

below 2 angstroms, which is significant for proteins with minimal amino acid

sequence conservation. This superpositioning based on 18 structurally conserved

amino acids allows detailed comparison of the structure of SPE-A with the

staphylococcal superantigens.

10   The crystal structure of the complex of staphylococcal superantigen SEB and

the class II major histocompatibility complex (MHC-II) shows amino acids on SEB

that contact MHC-II, including those listed on Table B. Superposition of the SPE-A

structure indicates the location of the amino acids of SPE-A that contact MHC-II,

contact residues or contact areas, in a complex of these two proteins. These

15   locations are shown in Figure 10 as balls.

Specifically, Figure 10 shows SPE-A 1 with B domain 2 including β-barrel

3, which is made up of various strands and loops. Locations 4-7 are on strand 8.

Location 4 is a distance equivalent to about 3 amino acids from the carboxy terminus

of strand 8, which is at the junction of strand 8 and loop 9, and just below a turn on

20   strand 8, by the orientation of Figure 10. Location 4 can be occupied by a polar

amino acid, preferably residue Asn-49 of SPE-A. Location 5 is near the center of

strand 4, and can be occupied by a hydrophobic amino acid, preferably residue Ile-

47 of SPE-A. A distance of about 1 amino acid intervenes between locations 5 and

6. Location 6 can be occupied by a hydrophobic amino acid, preferably Leu-41 of

25   SPE-A. Location 7 is at the amino terminal end of strand 8. Location 7 can be

occupied by a hydrophobic amino acid, preferably by Leu-42 of SPE-A.

Locations 10-12 are on strand 13 of β-barrel 3. Location 10 is about three

amino acids distant from the junction of loop 9 and strand 13, and there is a turn

between location 10 and that junction. Location 10 can be occupied by a charged

30   amino acid, preferably Lys-57 of SPE-A. Location 11 is near the center of strand

13, and can be occupied by a charged amino acid, preferably Lys-58 of SPE-A.

Location 11 is proximal to location 5. Location 12 is nearest the junction of loop 14

and strand 13, but on strand 13, and can be occupied by a charged amino acid, preferably residue Glu-61 of SPE-A. Location 53 is at the junction of strand 13 and loop 14. Location 13 can be occupied by a hydrophobic amino acid, preferably Leu-62 of SPE-A.

5 Locations 15, 16, and 17 are on loop 18. Location 15 is on loop 18 at a position where loop 18 crosses a plane defined by strands 8 and 13. Location 15 is adjacent to the center turn 26 of alpha helix 25. Location 15 can be occupied by an unchanged or a polar amino acid, preferably Ser-43 of SPE-A. Location 16 is on loop 18 at a point where loop 18 has risen above strand 8, as shown in Figure 10.

10 Location 16 can be occupied by a polar amino acid, preferably His-44 of SPE-A. Location 17 is on loop 18 proximal to locations 6 and 5. Location 17 can be occupied by a polar amino acid, preferably Gln-40 of SPE-A.

Locations 19-21 are on loop 22. These locations are separated by the distance of about a single amino acid. Locations 19 and 20 are approximately

15 midway along the length of loop 22. Location 19 can be occupied by a neutral or polar amino acid, preferably by Cys-90 of SPE-A. Location 20 can be occupied by a neutral or polar amino acid, preferably Tyr-88 of SPE-A. Location 21 can be occupied by a hydrophobic amino acid, preferably Leu-86 of SPE-A.

Location 23 is on α-helix 24 in the turn nearest the junction of helix 24 and

20 loop 18. Location 23 can be occupied by a polar amino acid, preferably His-44 of SPE-A.

The crystal structure of the complex of staphylococcal SEC and the T-cell receptor shows amino acids on SEC 3 that contact the T-cell receptor including residues listed in Table C. Super position of the SPE-A structure indicates the

25 location of the amino acids of SPE-A that contact the T-cell receptor in a complex of these two proteins. These locations are shown in Fig. 11 as balls.

Specifically, with reference to Fig. 11, these include locations 10-12 described hereinabove. Locations 27-30 are on loop 22. Each location is adjacent to the preceding location. Location 30 is at the junction of loop 22 and strand 31.

30 Location 27 can be occupied by a polar amino acid, preferably Asn-92 of SPE-A. Location 28 can be occupied by a neutral amino acid, preferably Ala-93 of SPE-A.

20

Location 29 can be occupied by a charged amino acid, preferably Glu-94 of SPE-A.

Location 30 can be occupied by a charged amino acid, preferably Arg-95 of SPE-A.

Location 32 is on strand 9, at about the middle of strand 9. Location 32 can

be occupied by a polar amino acid, preferably Asn-54 of SPE-A. Location 33 is at

5   the junction of loop 22 and strand 34. Location 33 can be occupied by a polar amino

acid, preferably Tyr-84 of SPE-A. Location 35 is on loop 22 adjacent to location 33.

Location 35 can be occupied by a polar amino acid, preferably His-85 of SPE-A.

Locations 36-40 are in N-terminal α-helix 41. Location 36 is on loop 42 of

N-terminal α-helix 41. Location 36 is the distance of one residue removed from the

10   junction of N-terminal α-helix 41 with loop 44. Locations 37-40 are adjacent

locations in loop 43 of N-terminal alpha helix 41. Location 36 can be occupied by a

hydrophobic amino acid, preferably Phe-23 of SPE-A. Location 37 can be occupied

by a polar amino acid, preferably Asn-20 of SPE-A. Location 38 can be occupied by

a polar amino acid, preferably Gln-19 of SPE-A. Location 39 can be occupied by a

15   hydrophobic amino acid, preferably Leu-18 of SPE-A. Location 40 can be occupied

by a polar amino acid, preferably Asn-17 of SPE-A.

Locations 45 and 46 are in a region of loop 47 proximal to turn 43 of the N-

terminal α-helix 41. Location 45 can be occupied by a polar amino acid, preferably

Tyr-160 of SPE-A. Location 46 can be occupied by a polar amino acid, preferably

20   Asn-162 of SPE-A. Locations 45 and 46 are separated by approximately the

distance of one amino acid residue.

Interactions between SPE-A and the liver renal tubular receptor includes

interactions with central α-helix 48 shown in Fig. 12. Locations on central α-helix

48 important to interaction with the liver receptor include locations 49-54.

25   Locations 49-55 define a surface of the central α-helix 45 that forms the base of a

groove in the structure of SPE-A. Locations 49-54 are preferred locations on this

surface. Locations 49 and 51 can be occupied by polar residues. Locations 50 and

52-54 can be occupied by charged residues. Preferably location 49 is Asn-156,

location 50 is amino acid Asp-55, location 51 is amino acid Tyr-152, location 52 is

30   amino acid Lys-151, location 53 is amino acid Lys-148, or location 54 is amino acid

Glu-144. More preferred locations are 50, 51, 53, and 54, which have the greatest

proportion of the location on the surface defined by locations 49-55. Location 55 is

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proximal to the junction of loop 56 and central α-helix 45. Location 55 can be occupied by a neutral or polar amino acid, preferably by Thr-141 of SPE-A.

Table B lists residues of SEB that interact with class 2 MHC in the crystal structure of the complex of these two proteins. Superposition of the structures of

5   SEC-3, SEA and TSST-1 with the structure of the SEB:MHC-II complex indicates amino acids on these proteins that correspond to the SEB residues that interact with MHC-II, including residues on these proteins listed in Table B. Preferred SPE-A mutants include substitution of an SPE-A residue that corresponds to a residue in SEB, SEC-3, SEA or TSST-1 that interacts with MHC-II. These preferred SPE-A

10   residues include the SPE-A residues listed in Table B. Corresponding residues from the different proteins are listed across the rows of the table.

Table C lists residues of SEC-3 that interact with the T-cell receptor in the crystal structure of the complex of these two proteins. Superposition of the structures of SEC-3, SEA and TSST-1 with the structure of the SEB:T-cell receptor

15   complex indicates amino acids on these proteins that correspond to the SEB residues that interact with T-cell receptor and inlcudes residues listed in Table C. Preferred SPE-A mutants include substitution of an SPE-A residue that corresponds to a residue in SEB, SEC-3, SEA or TSST-1 that interact with T-cell receptor. These preferred SPE-A residues include the SPE-A residues listed in Table B.

20   Corresponding residues from the different proteins are listed across the rows of the table.

Preferred mutants of SPE-A have amino acid substitutions in at least one of the locations or for at least one of the amino acid residues that interacts with the T-cell receptor, MHC-II or the liver neutral tubular cell receptor. These amino acid

25   substitutions can be chosen as described hereinabove to disrupt the interactions.

TABLE A

PTSAG CONSERVED RESIDUES

TSST-1	SEA	SEB	SEC-3	SPE-A
TYR 13	TYR 30	TYR 28	TYR 28	TYR 25
ASP 27	ASP 45	ASP 42	ASP 42	ASP 39
LYS 58	LYS 81	LYS 78	LYS 78	LYS 72
VAL 62	VAL 85	VAL 82	VAL 82	VAL 76
ASP 63	ASP 86	ASP 83	ASP 83	ASP 77
GLY 87	GLY 110	GLY 117	GLY 114	GLY 102
THR 89	THR 112	THR 119	THR 116	THR 104
LYS 121	LYS 147	LYS 152	LYS 151	LYS 137
LYS 122	LYS 148	LYS 153	LYS 152	LYS 138
LEU 129	LEU 155	LEU 160	LEU 159	LEU 145
ASP 130	ASP 156	ASP 161	ASP 160	ASP 146
ARG 134	ARG 160	ARG 162	ARG 161	ARG 150
LEU 137	LEU 163	LEU 168	LEU 167	LEU 153
LEU 143	LEU 169	LEU 171	LEU 170	LEU 159
TYR 144	TYR 170	TYR 172	TYR 171	TYR 160
GLY 152	GLY 182	GLY 185	GLY 184	GLY 170
ASP 167	ASP 197	ASP 199	ASP 199	ASP 185
ILE 189	ILE 226	ILE 230	ILE 230	ILE 214

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TABLE B

RESIDUES INVOLVED IN CLASS II MHC INTERACTIONS

SEB	TSST-1	SEA	SEC-3	SPE-A
Gln 43	Asn 28	Gln 46	Lys 43	Gln 40
Phe 44	Ser 29	Phe 47	Phe 44	Leu 41
Leu 45		Leu 48	Leu 45	Leu 42
Tyr 46	Leu 30	Gln 49	Ala 46	Ser 43
Phe 47	Gly 31	His 50	His 47	His 44
				Asp 45
Gln 92	Lys 71	Gln 95	Asn 92	Leu 86
Tyr 94	Gln 73	Ala 97	Tyr 94	Tyr 88
Ser 96		Gly 99	Ser 96	Cys 90
Met 215	Asn 175	Arg 211	Met 215	Met 199

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TABLE C

RESIDUES INVOLVED IN TCR INTERACTIONS

TSST-1	SEC-3	SEA	SPE-A	SEB
ASN 5	GLY 19	THR 21	ASN 17	GLY 19
	THR 20	ALA 22	LEU 18	LEV 20
ASP 8	ASN 23	ASN 25	ASN 20	ASN 23
ASP 11	TYR 26	GLN 28	PHE 23	VAL 26
	ASN 60		ASN 54	
LYS 70	TYR 90	GLY 93	TYR 84	TYR 90
	VAL 91	TYR 94	HIS 85	TYR 91
	GLY 102		ASN 92	
	LYS 103		ALA 93	
	VAL 104		GLU 94	
	SER 106	LYS 103	ARG 95	
ARG 145	PHE 176	ASN 171	ASN 162	TYR 175
	GLN 210	SER 206	GLN 194	GLN 210

25

Mutant SPE-A toxins with changes to cysteine residues or introduction of disulfide bonds can be selected that have a decrease in lethality, or optionally a decrease in enhancement of endotoxin shock, and/or a decrease in T cell mitogenicity. A specific example is change at the cysteine residue 98. A change at

5 this residue from cysteine to serine results in a mutant toxin with a decrease in mitogenicity about four-fold and a decrease in enhancement in endotoxin shock and a decrease in lethality due to STSS. Changes that eliminate the cysteine group at residue 98 can effect biological activity in a similar manner as a substitution with serine. Other changes that could be made at residue 98 include substitution of the

10 other small aliphatic residues such as alanine, glycine or threonine. Changes at other cysteine residues at amino acid residues 90 and 97 result in a decrease in mitogenicity.

Advantageously, mutant SPE-A toxins useful in treatment methods can be generated that have more than one change in the amino acid sequence. It would be

15 desirable to have changes at more than one location to minimize any chance of reversion to a molecule having toxicity or lethality. For vaccine compositions, it is desirable that a mutant toxin with multiple changes can still generate a protective immune response against wild type SPE-A and/ or immunoreact with neutralizing polyclonal antibodies to wild type SPE-A. For pharmaceutical compositions, it is

20 preferred that mutants with multiple changes are substantially nonlethal while maintaining mitogenicity for T cells. It is especially preferable to have about 2 to 6 changes. Examples of such mutants include those with the N20D mutation including double mutants such as N20D/K157E, N20D/C98S, triple mutants, such as N20D/D45N/C98S, and the like. Double mutant N20D/C98S has been deposited

25 with the ATCC and has accession no. 55821. Double mutant N20D/C98S has been deposited with the ATCC and has accession no. 55822. Triple mutant N20D/D45N/C98S has been deposited with the ATCC and has accession no. 55993.

Double mutants of SPE A may offer advantages over single mutants. This was evaluated in three experiments detailed in Example 6. Results are provided in

30 Figures 7-9. The data indicated that the N20D/C98S mutant had less toxicity than the single N20D mutant and the double mutant N20D/K157E was intermediate between the other two proteins. All three mutants were significantly less toxic than

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wild type SPE A. Sera from rabbits immunized with the single and double mutants
inhibited lymphocyte proliferation in response to nonmutated SPE A toxin.
Lymphocyte proliferation is associated with and necessary for full toxicity of the
toxin.

5   Animals were immunized against N20D, N20D/C98S, or N20D/K157E, as
described in Example 7. Results are provided in Table 9. Animals immunized with
either double mutant were completely protected from fever and enhanced
susceptibility to endotoxin shock.

          Triple mutants are also contemplated in this application and in one
10   embodiment, the SPE-A mutant N20D/C98S/D45N was tested using the methods
and assays of Examples 1-7 and the primers disclosed herein.

          It may also be preferable to delete residues at specific sites such as deletion
of amino acid residue 20 asparagine and/or deletion of amino acid 157 lysine or 98
cysteine. For vaccine compositions, mutants with deletions would be selected that
15   immunoreact with polyclonal neutralizing antibodies to wild type SPE-A toxin
and/or can stimulate a protective immune response against wild type SPE-A activity.

          Mutant toxins of SPE-A are useful to form vaccine compositions. The
preferred mutants for vaccine compositions have at least one amino acid change, are
nontoxic systemically, and immunoreact with polyclonal neutralizing antibodies to
20   wild type SPE-A. The especially preferred mutants include those mutant SPE-A
toxins with a change at amino acid 20 such as N20D, N20D/K157E, N20D/C98S,
and mutants with a deletion at residue 20 asparagine.

          Mutant toxins are combined with a physiologically acceptable carrier.
Physiologically acceptable diluents include physiological saline solutions, and
25   buffered saline solutions at neutral pH such as phosphate buffered saline. Other
types of physiological carriers include liposomes or polymers and the like.
Optionally, the mutant toxin can be combined with an adjuvant such as Freund's
incomplete adjuvant, Freund's Complete adjuvant, alum, monophosphoryl lipid A,
alum phosphate or hydroxide, QS-21 and the like. Optionally, the mutant toxins or
30   fragments thereof can be combined with immunomodulators such as interleukins,
interferons and the like. Many vaccine formulations are known to those of skill in
the art.

The mutant SPE-A toxin or fragment thereof is added to a vaccine formulation in an amount effective to stimulate a protective immune response in an animal to at least one biological activity of wild type SPE-A toxin. Generation of a protective immune response can be measured by the development of antibodies,

5 preferably antibodies that neutralize the wild type SPE-A toxin. Neutralization of wild type SPE-A toxin can be measured including by inhibition of lethality due to wild type SPE-A in animals. In addition, a protective immune response can be detected by measuring a decrease in at least one biological activity of wild type SPE-A toxins such as amelioration or elimination of the symptoms of enhancement of

10 endotoxin shock or STSS. The amounts of the mutant toxin that can form a protective immune response are about 0.1 µg to 100 mg per kg of body weight more preferably about 1 µg to about 100 µg/kg body weight. About 25 µg/kg of body weight of wild type SPE-A toxin is effective to induce protective immunity in rabbits.

15   The vaccine compositions are administered to animals such as rabbits, rodents, horses, and humans. The preferred animal is a human.

The mutant SPE-A toxins are also useful to form pharmaceutical compositions. The pharmaceutical compositions are useful in therapeutic situations where a stimulation of T-cell proliferation may be desirable, such as in the treatment

20 of cancer. The preferred mutant SPE-A toxins are those that are nonlethal while maintaining T-cell mitogenicity comparable to wild type SPE-A toxin. Preferred mutants are those that have a change at residue 157 lysine of wild type SPE-A toxins such as K157E.

A pharmaceutical composition is formed by combining a mutant SPE-A

25 toxin with a physiologically acceptable carrier such as physiological saline, buffered saline solutions at neutral pH such as phosphate buffered saline. The mutant SPE-A toxin is combined in an amount effective to stimulate T-cell proliferation comparable to wild type SPE-A toxin at the same dose. An enhancement in T-cell responsiveness can be measured using standard 3H thymidine assays with rabbit

30 lymphocytes as well as by measuring T-cell populations in vivo using fluorescence activated T-cell sorters or an assay such as an ELISPOT. An effective amount can also be an amount effective to ameliorate or decrease the growth of cancer cells.

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This can be determined by measuring the effect of the mutant SPE-A toxin on growth of cancer cells in vivo or by measuring the stimulation of cancer-specific T-cells. The range of effective amounts are 100 ng to 100 mg per kg of body weight, more preferably 1 µg to 1 mg/kg body weight. About 10-6 µg of wild type SPE-A

5   toxin can stimulate enhanced T cell responsiveness. For example, these mutant SPE-A toxins could be used either alone or in conjunction with interleukin or interferon therapy.

The invention also includes fragments of SPE-A toxins and fragments of mutant SPE-A toxins. For vaccine compositions, fragments are preferably large

10   enough to stimulate a protective immune response. A minimum size for a B cell epitope is about 4-7 amino acids and for a T cell epitope about 8- 12 amino acids. The total size of wild type SPE-A is about 251 amino acids including the leader sequence. Fragments are peptides that are about 4 to 250 amino acids, more preferably about 10-50 amino acids.

15   Fragments can be a single peptide or include peptides from different locations joined together. Preferably, fragments include one or more of the domains as identified in Figure 1 and as described previously. It is also preferred that the fragments from mutant SPE-A toxins have at least one change in amino acid sequence and more preferably 1-6 changes in amino acid sequence when compared

20   to a protein substantially corresponding to a wild type SPE-A toxin.

Preferably, fragments are substantially nonlethal systemically. Fragments are screened and selected to have little or no toxicity in rabbits using the miniosmotic pump model at the same or greater dosage than a protein having wild type SPE-A toxin activity as described previously. It is also preferred that the

25   fragment is nontoxic in humans when given a dose comparable to that of a wild type SPE-A toxin.

For vaccine compositions, it is preferred that the fragments include residues from the central a helix and/or the N-terminal a helix. It is especially preferred that the fragment include a change at amino acid residues equivalent to residue 20 in

30   wild type SPE-A toxin such as N20D or a change at an amino acid residue equivalent to residue 98 cysteine in a wild type SPE-A toxin.

29

For vaccine compositions, it is preferable that a fragment stimulate a neutralizing antibody response to a protein having wild type SPE-A toxin activity. A fragment can be screened and selected for immunoreactivity with polyclonal neutralizing antibodies to a wild type SPE-A toxin. The fragments can also be used

5 to immunize animals and the antibodies formed tested for neutralization of wild type SPE-A toxin.

For vaccine compositions, especially preferred fragments are further selected and screened to be nonbiologically active. By nonbiologically active, it is meant that the fragment is nonlethal systemically, induces little or no enhancement of

10 endotoxin shock, and induces little or no T cell stimulation. Optionally, the fragment can be screened and selected to have a decrease in capillary leak effect on porcine endothelial cells.

The fragments screened and selected for vaccine compositions can be combined into vaccine formulations and utilized as described previously.

15 Optionally, fragments can be attached to carrier molecules such as bovine serum albumin, human serum albumin, keyhole limpet hemocyanin, tetanus toxoid and the like:

For pharmaceutical compositions, it is preferred that the fragments include amino acid residues in the N-terminal Domain B β strands 1-5 alone or in

20 combination with the central a helix. It is especially preferred if the fragments include a change at an amino acid residue equivalent to the lysine at amino acid 157 of a wild type SPE-A toxin such as K157E.

For pharmaceutical compositions, it is preferred that the fragments are screened and selected for nonlethality systemically, and optionally for little or no

25 enhancement of endotoxin shock as described previously. It is preferred that the fragments retain T cell mitogenicity similar to the wild type SPE-A toxin. Fragments of a mutant toxin SPE-A can form pharmaceutical compositions as described previously.

Fragments of mutant SPE-A toxin can be prepared using PCR, restriction

30 enzyme digestion and/or ligation, in vitro mutagenesis and chemical synthesis. For smaller fragments chemical synthesis may be desirable.

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The fragments of mutant SPE-A toxins can be utilized in the same compositions and methods as described for mutant SPE-A toxins.

B.   Methods for using mutant SPE-A toxins, vaccines compositions or pharmaceutical compositions.

The mutant SPE-A toxins and/ or fragments thereof are useful in methods for protecting animals against the effects of wild type SPE-A toxins, ameliorating or treating animals with STSS, inducing enhanced T-cell proliferation and responsiveness, and treating or ameliorating the symptoms of cancer.

A method for protecting animals against at least one biological activity of wild type SPE-A toxin involves the step of administering a vaccine composition to an animal to establish a protective immune response against at least one biological activity of SPE-A toxin. It is preferred that the protective immune response is neutralizing and protects against lethality or symptoms of STSS. The vaccine composition preferably includes a mutant SPE-A toxin or fragment thereof that has at least one amino acid change, that immunoreacts with polyclonal neutralizing antibodies to wild type SPE-A, and is nonlethal. The especially preferred mutant has a change at amino acid residue 20 asparagine such as the mutant N20D, or N20D/K157E or N20D/C98S.

The vaccine composition can be administered to an animal in a variety of ways including subcutaneously, intramuscularly, intravenously, intradermally, orally, intranasally, ocularly, intraperitoneally and the like. The preferred route of administration is intramuscularly.

The vaccine compositions can be administered to a variety of animals including rabbits, rodents, horses and humans. The preferred animal is a human.

The vaccine composition can be administered in a single or multiple doses until protective immunity against at least one of the biological activities of wild type SPE-A is established. Protective immunity can be detected by measuring the presence of neutralizing antibodies to the wild type SPE-A using standard methods.

An effective amount is administered to establish protective immunity without causing substantial toxicity.

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A mutant SPE-A toxin or fragment thereof is also useful to generate neutralizing antibodies that immunoreact with the mutant SPE-A toxin and the wild type SPE-A toxin. These antibodies could be used as a passive immune serum to treat or ameliorate the symptoms in those patients that have the symptoms of STSS.

5   A vaccine composition as described above could be administered to an animal such as a horse or a human until a neutralizing antibody response to wild type SPE-A is generated. These neutralizing antibodies can then be harvested, purified, and utilized to treat patients exhibiting symptoms of STSS. Neutralizing antibodies to wild type SPE-A toxin can also be formed using wild type SPE-A. However, wild

10   type SPE-A must be administered at a dose much lower than that which induces toxicity such as 1/50 to 1/100 of the LD50 of wild type SPE-A in rabbits.

The neutralizing antibodies are administered to patients exhibiting symptoms of STSS such as fever, hypotension, group A streptococcal infection, myositis, fascitis, and liver damage in an amount effective to neutralize the effect of SPE-A

15   toxin. The neutralizing antibodies can be administered intravenously, intramuscularly, intradermally, subcutaneously, and the like. The preferred route is intravenously or for localized infection, topically at the site of tissue damage with debridement. It is also preferred that the neutralizing antibody be administered in conjunction with antibiotic therapy. The neutralizing antibody can be administered

20   until a decrease in shock or tissue damage is obtained in a single or multiple dose. The preferred amount of neutralizing antibodies typically administered is about 1mg to 1000mg/kg, more preferably about 50-200mg/kg of body weight.

The mutant SPE-A toxins and/ or fragments thereof are also useful in pharmaceutical compositions for stimulation of T-cell proliferation, especially in the

25   treatment of cancer. It is especially preferred that these pharmaceutical compositions be used in the place of or in conjunction with current therapies for cancer using interleukins, interferons or tumor necrosis factors. The mutant SPE-A toxins are also useful in treating T cell lymphomas, and ovarian and uterine cancer. While not meant to limit the invention, it is believed that mutant SPE-A toxins can

30   be selectively toxic for T lymphoma cells.

The pharmaceutical compositions include a mutant SPE-A toxin and/ or fragment thereof that are nonlethal, while maintaining T cell mitogenicity. The

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preferred mutant SPE-A toxin is one that has a change at amino acid residue 157 lysine such as K157E.

The pharmaceutical composition is administered to a patient having cancer by intravenous, intramuscular, intradermal, orally, intraperitoneally, and

5   subcutaneous routes, and the like. The preferred route is intravenous. The pharmaceutical composition can be administered in a single dose or multiple doses. The pharmaceutical composition is administered in an amount that is effective to stimulate enhanced T-cell proliferative response and/or to decrease the growth of the cancer without substantial toxicity. The preferred amount ranges from 100 ng to

10   100 mg/kg, more preferably 1 µg to 1 mg/kg. It is especially preferred that the mutant SPE-A pharmaceutical compositions are administered in conjunction with or in place of therapies using interferons, interleukins, or tumor necrosis factors.

C.   DNA Expression Cassettes Encoding Mutant SPE-A Toxins and

15   **Methods of Preparation of Such DNA Expression Cassettes**

The invention also includes DNA sequences and expression cassettes useful in expression of mutant SPE-A toxins and/or fragments thereof. An expression cassette includes a DNA sequence encoding a mutant SPE-A toxin and/ or fragment thereof with at least one amino acid change and at least one change in biological

20   function compared to a protein substantially corresponding to a wild type SPE-A toxin operably linked to a promoter functional in a host cell. Expression cassettes are incorporated into transformation vectors and mutant SPE-A toxins are produced in transformed cells. The mutant toxins can then be purified from host cells or host cell supernatants. Transformed host cells are also useful as vaccine compositions.

25   Mutant SPE-A toxins or fragments thereof can also be formed by screening and selecting for spontaneous mutants in a similar manner as described for site specific or random mutagenesis. Mutant SPE-A toxins can be generated using in vitro mutagenesis or semisynthetically from fragments produced by any procedure. Finally, mutant SPE-A toxins can be generated using chemical synthesis.

30   A method of producing the mutant SPE-C toxins or fragments thereof which includes transforming or transfecting a host cell with a vector including such an

33

expression cassette and culturing the host cell under conditions which permit

expression of such mutant SPE-C toxins or fragments by the host cell.

DNA Sequences Encoding Mutant SPE-A Toxins

5   A mutant DNA sequence encoding a mutant SPE-A toxin that has at least

one change in amino acid sequence can be formed by a variety of methods

depending on the type of change selected. A DNA sequence encoding a protein

substantially corresponding to wild type SPE-A toxin functions as template DNA

used to generate DNA sequences encoding mutant SPE-A toxins. A DNA sequence

10   encoding wild type SPE-A toxin is shown in Figure 3 and has been deposited in a

microorganism with ATTC Accession number 69830.

       To make a specific change or changes at a specific location or locations it is

preferred that PCR is utilized according to method of Perrin et al., cited supra. To

target a change to a particular location, internal primers including the altered

15   nucleotides coding for the amino acid change are included in a mixture also

including a 5' and 3' flanking primers. A 5' flanking primer is homologous to or

hybridizes to a DNA region upstream of the translation start site of the coding

sequence for wild type SPE-A. Preferably, the 5' flanking region is upstream of the

speA promoter and regulatory region. For example, a 5' flanking primer can be

20   homologous to or hybridize to a region about 760 bases upstream of the translation

start site as shown in Figure 2. An example of a 5' flanking primer which includes

the SPE-A promoter in upstream regulatory region has a sequence of:

```
            5' GGT GGA TTC TTG AAA CAG
                   BamH1
25             GTG-3' (SEQ ID NO:1)
```

A downstream flanking primer is homologous to or hybridizes to a region of DNA

downstream of the stop codon of the coding sequence for wild type SPE-A. It is

preferred that the downstream flanking primer provides for transcriptional and

translational termination signals. For example, a 3' flanking primer can hybridize or

30   be homologous to a region 200 base pairs downstream of the stop codon for the

coding sequence of SPE-A. An example of a 3' flanking primer has a sequence:

```
            5' CCC CCC GTC GAC GAT AAA ATA GTT GCT
                          SalI
            AAG CTA CAA GCT-3' (SEQ ID NO:2)
```

34

The upstream and downstream flanking primers are present in every PCR reaction to
ensure that the resulting PCR product includes the speA promoter and upstream
regulatory region and transcriptional and translation termination signals. Other

5   upstream and downstream primers can readily be constructed by one of skill in the
art. While preferred, it is not absolutely necessary that the native *speA* promoter and
upstream regulatory region be included in the PCR product.

Each mutation at a particular site is generated using an internal primer
including a DNA sequence coding for a change at a particular residue. For example,

10   amino acid substitutions at a specific site can be generated using the following
internal primers:

Mutant	Internal Primer
N20D	5' AAA AAC CTT CAA GAT ATA TAT TTT CTT -3' (SEQ ID NO:3)
C87S	5'-TCC-ACA-TAA-ATA GCT GAG ATG GTA ATA-TCC-3' (SEQ ID NO:4)
C90S	5'-CTC TGT TAT TTA TCT GAA AAT GCA GAA-3' (SEQ ID NO:5)
C98S	5' CCC TCC GTA GAT CGA TGC ACT CCT TTC TGC-3' (SEQ ID NO:6)
K157E	5'-CTT ACA GAT AAT GAG CAA CTA TAT ACT-3' (SEQ ID NO:7)
S195A	5'-CCA GGA TTT ACT CAA GCT AAA TAT CTT ATG-3' (SEQ ID NO:8)
K16N	5'- CAA CTT CAC AGA TCT AGT TTA GTT AAC AAC CTT-3' (SEQ ID NO:9) (forward primer) and
	5'- T TTG AAG GTT GTT AAC TAA ACT AGA TCT GTG AAG TTG-3' (backward primer)(SEQ ID NO:10)

40   The underlined nucleotides indicate changes in the nucleotide sequence from a wild
type *speA* gene as shown in Figure 3.

Internal primers can be designed to generate a change at a specific location
utilizing a DNA sequence encoding wild type SPE-A toxins such as shown in Figure
3. Primers can be designed to encode a specific amino acid substitution at a specific

35

location such as shown above. Primers can be designed to result in random

substitution at a particular site as described by Rennell et al., J. Mol. Biol. 22:67

(1991). Primers can be designed that result in a deletion of an amino acid at a

particular site. Primers can also be designed to add coding sequence for an

5   additional amino acid at a particular location.

Primers are preferably about 15 to 50 nucleotides long, more preferably 15 to

30 nucleotides long. Primers are preferably prepared by automated synthesis. The

5' and 3' flanking primers preferably hybridize to the flanking DNA sequences

encoding the coding sequence for the wild type SPE-A toxin. These flanking

10   primers preferably include about 10 nucleotides that are 100% homologous or

complementary to the flanking DNA sequences. Internal primers are not 100%

complementary to DNA sequence coding for the amino acids at location because

they encode a change at that location. An internal primer can have about 1 to 4

mismatches from the wild type SPE-A sequence in a primer about 15 to 30

15   nucleotides long. Both flanking primers and internal primers can also include

additional nucleotides that encode for restriction sites and clamp sites, preferably

near the end of the primer. Hybridization conditions can be modified to take into

account the number of mismatches present in the primer in accord with known

principles as described by Sambrook et al. Molecular Cloning-A laboratory manual,

20   Cold Spring Harbor Laboratory Press, (1989).

More than one internal primer can be utilized if changes at more than one site

are desired. For example, to generate a mutant having a change at amino acid 20

asparagine and a change at amino acid 157 lysine internal primers as shown above

can be utilized in two separate reactions as described in Example 5. A PCR method

25   for generating site-specific changes at more than one location is described in Aiyar

et al. cited supra. Another method is described in Example 5.

In one method, a DNA sequence encoding a mutant SPE-A toxin with one

change at a particular site is generated and is then used as the template to generate a

mutant DNA sequence with a change at a second site. In the first round of PCR, a

30   first internal primer is used to generate the mutant DNA sequence with the first

change. The mutant DNA sequence with the first change is then used as the

template DNA and a second internal primer coding for a change at a different site is

used to form a DNA sequence encoding a mutant toxin with changes in amino acid sequences at two locations. PCR methods can be utilized to generate DNA sequences with encoding amino acid sequences with about 2 to 6 changes.

The preferred PCR method is as described by Perrin et al. cited supra.

5   Briefly, the PCR reaction conditions are: PCR is performed in a 100 ul reaction mixture containing 10 mM Tris-HCl (pH=8.3), 50 mM KCl, 1.5 mM MgCl2, 200 uM each dNTP, 2 ng template plasmid DNA, 100 pmoles flanking primer, 5 pmoles internal primer, and 2.5 units of Ampli Taq DNA polymerase (Perkin Elmer Cetus) . In the second amplification step, the composition of the reaction mix is as above

10   except for equal molarity (5 pmoles each) of flanking primer and megaprimer and 1ug template. PCR is conducted for 30 cycles of denaturation at 94°C X 1 minute, annealing at 37°C or 44°C X 2minutes and elongation at 72°C for 3 minutes.

The PCR products are isolated and then cloned into a shuttle vector (such as pMIN 164 as constructed by the method of Murray et al, J. Immunology 152:87

15   (1994) and available from Dr. Schlievert, University of Minnesota, Mpls, MN.). This vector is a chimera of E. coli plasmid pBR328 which carries ampicillin resistance and the staphylococcal plasmid pE194 which confers erythromycin resistance. The ligated plasmid mixtures are screened in E. coli for toxin production using polylconal neutralizing antibodies to wild type SPE-A from Toxin

20   Technologies, Boca Raton, Fla or from Dr. Schlievert. The mutant SPE-A toxins are sequenced by the method of Hsiao et al., Nucleic Acid Res. 19:2787 (1991) to confirm the presence of the desired mutation and absence of other mutations.

Specific DNA sequences generated in this manner include a DNA sequence that encodes mutant N20D and has the same coding sequence as shown in Figure 3

25   except that an adenine at position 939 is changed to a guanine residue. A DNA sequence that encodes mutant C87S has the same coding sequence of Figure 3 except that thymine at position 1,152 is changed to a adenine and thymine at position 1,154 is changed to cytosine. A DNA sequence that encodes mutant SPE-A toxin C98S has the same coding sequence as Figure 3 except that guanine at position

30   1,185 is changed to cytosine and thymine at position 1,186 is changed to guanine. A DNA sequence that encodes mutant SPE-A toxin C90S includes a sequence that has the same coding sequence as Figure 3 except that guanine at position 1,161 is

57

changed to a cytosine. A DNA sequence that encodes mutant SPE-A toxin K157E includes a sequence that is the same as the coding sequence shown in Figure 3 but is changed at position 1,351 from adenine to guanine. A DNA sequence that encodes a mutant SPE-A toxin S195A includes a DNA sequence that has the same coding sequence as shown in Figure 3 except that thymine at position 1,464 is a guanine. A DNA sequence that encodes a mutant K16N SPE-A toxin includes a sequence that is the same as that shown in Figure 3 except that adenine at position 941 is changed to cytosine.

It will be understood by those of skill in the art that due to the degeneracy of the genetic code a number of DNA sequences can encode the same changes in amino acids. The invention includes DNA sequences having different nucleotide sequences but that code for the same change in amino acid sequence.

For random mutagenesis at a particular site a series of primers are designed that result in substitution of each of the other 19 amino acids or a non-naturally occurring amino acid or analog at a particular site. PCR is conducted in a similar manner as described above or by the method described by Rennell et al., cited supra. PCR products are subcloned and then toxin production can be monitored by immunoreactivity with polylconal neutralizing antibodies to wild type SPE-A. The presence of a change in amino acid sequence can be verified by sequencing of the DNA sequence encoding the mutant SPE-A toxin. Preferably, mutant toxins are screened and selected for nonlethality.

Other methods of mutagenesis can also be employed to generate random mutations in the DNA sequence encoding the wild type SPE-A toxin. Random mutations or random mutagenesis as used in this context means mutations are not at a selected site and/or are not a selected change. A bacterial host cell including a DNA sequence encoding the wild type SPE-A toxin, preferably on pMIN 164, can be mutagenized using other standard methods such as chemical mutagenesis, and UV irradiation. Mutants generated in this manner can be screened for toxin production using polyclonal neutralizing antibodies to wild type SPE-A. However, further screening is necessary to identify mutant toxins that have at least one change in a biological activity, preferably that are nonlethal. Spontaneously arising mutants

can also be screened for at least one change in a biological activity from wild type SPE-A.

Random mutagenesis can also be conducted using in vitro mutagenesis as described by Anthony-Cahill et al., Trends Biochem. Sci. 14: 400 (1989).

5   In addition, mutant SPE-A toxins can be formed using chemical synthesis. A method of synthesizing a protein chemically is described in Wallace, FASEB J. 7:505 (1993). Parts of the protein can be synthesized and then joined together using enzymes or direct chemical condensation. Using chemical synthesis would be especially useful to allow one of skill in the art to insert non-naturally occurring

10   amino acids at desired locations. In addition, chemical synthesis would be especially useful for making fragments of mutant SPE-A toxins.

Any of the methods described herein would be useful to form fragments of mutant SPE-A toxins. In addition, fragments could be readily generated using restriction enzyme digestion and/or ligation. The preferred method for generating

15   fragments is through direct chemical synthesis for fragment of 20 amino acids or less or through genetic cloning for larger fragments.

DNA sequences encoding mutant toxins, whether site-specific or random, can be further screened for other changes in biological activity from wild type SPE-A toxin. The methods for screening for a change in at least one biological activity

20   are described previously. Once selected DNA sequences encoding mutant SPE-A toxins are selected for at least one change in biological activity, they are utilized to form an expression cassette.

Formation of an expression cassette involves combining the DNA sequences coding for mutant SPE-A toxin with a promoter that provides for expression of a

25   mutant SPE-A toxin in a host cell. For those mutant SPE-A toxins produced using PCR as described herein, the native *speA* promoter is present and provides for expression in a host cell.

Optionally, the DNA sequence can be combined with a different promoter to provide for expression in a particular type of host cell or to enhance the level of

30   expression in a host cell. Preferably, the promoter provides for a level of expression of the mutant SPE-A toxin so that it can be detected with antibodies to SPE-A.

Other promoters that can be utilized in prokaryotic cells include PLAC, PTAC, T7, and the like.

Once the DNA sequence encoding the mutant SPE-A toxin is combined with a suitable promoter to form an expression cassette, the expression cassette is
5   subcloned into a suitable transformation vector. Suitable transformation vectors include at least one selectable marker gene and preferably are shuttle vectors that can be amplified in E. coli and gram positive microorganisms. Examples of suitable shuttle vectors include pMIN 164, and pCE 104. Other types of vectors include viral vectors such as the baculovirus vector, SV40, poxviruses such as vaccinia,
10   adenovirus and cytomegalovirus. The preferred vector is a pMIN 164 vector, a shuttle vector that can be amplified in E. coli and S. aureus.

Once a transformation vector is formed carrying an expression cassette coding for a mutant SPE-A toxin, it is introduced into a suitable host cell that provides for expression of the mutant SPE-A toxin. Suitable host cells are cells that
15   provide for high level of expression of the mutant toxin while minimizing the possibility of contamination with other undesirable molecules such as endotoxin and M-proteins. Suitable host cells include mammalian cells, bacterial cells such as S. aureus, E. coli and Salmonella spp., yeast cells, and insect cells.

Transformation methods are known to those of skill in the art and include
20   protoplast transformation, liposome mediated transformation, calcium phosphate precipitation and electroporation. The preferred method is protoplast transformation.

Preferred transformed cells carry an expression cassette encoding a mutant SPE-A toxin with a change at amino acid 20 asparagine. Such a transformed cell has been deposited with the American Type Culture Collection in Rockville,
25   Maryland. The characteristics of the deposited microorganism is that it is a S. aureus carrying pMIN 164 including a DNA sequence encoding mutant N20D operably linked to the native speA promoter and other regulatory regions. This microorganism was deposited in accordance with the Budapest treaty and given Accession number 69831.

30   Another microorganism has been deposited with the ATCC. This microorganism is S. aureus carrying a DNA sequence encoding the wild type SPE-A toxin operably linked to the native speA promoter and regulatory regions. This

40

microorganism was deposited with the ATCC in accord with the Budapest treaty and given Accession number 69830.

Transformed cells are useful to produce large amounts of mutant SPE-A toxin that can be utilized in vaccine compositions. A transformed microorganism

5   can be utilized in a live, attenuated, or heat killed vaccine. A transformed microorganism includes mutant toxin SPE-A in amounts sufficient to stimulate a protective immune response to wild type SPE-A. Preferably, the mutant SPE-A toxin is secreted. The microorganism is preferably nonpathogenic to humans and includes a mutant toxin with multiple amino acid changes to minimize reversion to a

10   toxic form. The microorganism would be administered either as a live or heat killed vaccine in accordance with known principles. Preferred microorganisms for live vaccines are transformed cells such as Salmonella spp.

A viral vector including an expression cassette with a DNA sequence encoding a mutant SPE- A toxin or fragment thereof operably linked to a promoter

15   functional in a host cell can also be utilized in a vaccine composition as described herein. Preferably, the promoter is functional in a mammalian cell. An example of a suitable viral vector includes pox viruses such as vaccinia virus, adenoviruses, cytomegaloviruses and the like. Vaccinia virus vectors could be utilized to immunize humans against at least one biological activity of a wild type SPE-A

20   toxin.

The invention also includes a vaccine composition comprising an nucleic acid sequence encoding a mutant SPE-A toxin or fragment thereof operably linked to a promoter functional in a host cell. The promoter is preferably functional in a mammalian host cell. The nucleic acid sequence can be DNA or RNA. The vaccine

25   composition is delivered to a host cell or individual for expression of the mutant SPE A toxin or fragment thereof within the individuals own cells. Expression of nucleic acid sequences of the mutant SPE A toxin or fragment thereof in the individual provides for a protective immune response against the wild type SPE A toxin. Optionally, the expression cassette can be incorporated into a vector. A

30   nucleic acid molecule can be administered either directly or in a viral vector. The vaccine composition can also optionally include a delivery agent that provides for delivery of the vaccine intracellularly such as liposomes and the like. The vaccine

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composition can also optionally include adjuvants or other immunomodulatory

compounds, and additional compounds that enhance the uptake of nucleic acids into

cells. The vaccine composition can be administered by a variety of routes including

parenteral routes such as intravenously, intraperitoneally, or by contact with mucosal

5   surfaces.

        Conditions for large scale growth and production of mutant SPE-A toxin are

known to those of skill in the art. A method for purification of mutant SPE-A toxins

from microbial sources is as follows. S. aureus carrying the mutant or the wild type

*speA*s in pMIN164 are grown at 37°C with aeration to stationary phase in dialyzable

10   beef heart medium, containing 5mg/ml of erythromycin. Cultures are precipitated

with four volumes of ethanol and proteins resolubilized in pyrogen free water. The

crude preparations are subjected to successive flat bed isoelectric focusing

separations in pH gradients of 3.5 to 10 and 4 to 6. The fractions that are positive

for toxin by antibody reactivity are extensively dialyzed against pyrogen free water,

15   and an aliquot of each is tested for purity by SDS polyacrylamide gel electrophoresis

in 15% (weight/volume) gels . Polyclonal neutralizing antibodies to SPE-A are

available from Toxin Technologies, Boca Raton, Fla or Dr. Schlievert. Other

methods of purification including column chromatography or HPLC can be utilized.

        This invention can be better understood by way of the following examples

20   which are representative of the preferred embodiments thereof, but which are not to

be construed as limiting the scope of the invention.

EXAMPLE 1
Cloning and Expression of SPE-A Wild Type

25   The gene encoding wild type SPE-A toxin (*speA*) was cloned from E. coli as

described in Johnson et al., Mol. Gen. Genet. 194:52-56 (1984). Briefly, the *speA*

gene was identified by cloning of a HindIII digest of Phage T12 DNA in pBR322 in

E. Coli RR1. Transformants were selected by identifying those positive for toxin

production using polylconal neutralizing antisera to A toxin. A nucleotide sequence

30   for A toxin is reported in Weeks et al, Inf. Imm. 52: 144 (1986).

        A DNA sequence including the *speA* gene was subcloned and then expressed

in S. aureus. The *speA* carried on a E. coli plasmid was digested with restriction

42

enzymes HindIII and SalI. The fragments were purified and ligated into HindIII-Sall sites of pMIN 164 (available as described previously). The vector pMIN 164 is a chimera of the staphylococcal plasmid pE194 (carrying erythromycin resistance) and the E. coli vector pBR328 (carrying Amp and Tet resistance). Cloning of *speA*

5 into the HindIII-SalI sites of this vector disrupts Tet resistance. The promoter present in this plasmid immediately upstream of the cloned gene is the native *speA* promoter.

Expression of the *speA* gene was verified by detecting the toxin in a double immunodiffusion assay with polyclonal neutralizing antibodies to SPE-A from

10 Toxin prepared in the inventors laboratory.

EXAMPLE 2
Administration and Immunization of Rabbits with Recombinantly Produced SPE-A (wt)

15

Administration of recombinantly produced SPE-A to animals induces STSS. Immunization of animals with recombinantly produced SPE-A reduces the death rate when animals are challenged with M3 or M1 streptococci and protects animals against STSS.

20 Administration of SPE-A induces STSS in rabbits. A rabbit model for STSS has been established by administration of SPE-A in subcutaneously implanted miniosmotic pumps. Lee et al., Infect Immun. 59:879 (1991). These pumps are designed to release a constant amount of toxin over a 7-day period, thus providing continuous exposure to the toxin. Recombinantly produced SPE-A was

25 administered to rabbits at a total dose of 200μg/in 0.2 ml over a 7-day period. The results indicate that animals treated with SPE-A developed the criteria of STSS with nearly all animals succumbing in the 7-day period (data not shown). The symptoms of STSS in rabbits include weight loss, diarrhea, mottled face, fever, red conjunctiva and mucosa, and clear brown urine. As expected, control non-toxin treated animals

30 remained healthy. Two other major observations were made: 1) fluid replacement provided complete protection to the animals as expected, and 2) none of the toxin treated animals developed necrotizing fascitis and myositis, indicating factors other than, or in addition to, SPE-A are required for the soft tissue damage.

43

Development of the clinical features of STSS correlates with administration of SPE-A. Rabbits injected with SPE-A positive streptococci developed STSS whereas those injected with SPE-A negative streptococci did not show symptoms of STSS.

5   It is well known that SPE-A is a variable trait made by some group A streptococci. The gene for SPE-A is encoded by bacteriophage T12, and well-characterized streptococcal strains were established that differ only in whether or not the SPE-A phage, referred to as T12 phage, is present. Streptococcal strain T253 cured T12 is positive for production of SPE-A, whereas T253 cured is SPE-A

10   negative.

Rabbits were injected subcutaneously with SPE-A positive streptococci T253 cured T12 or SPE-A negative T253 cured into implanted Wiffle golf balls, as described by Scott et al., Infect Immunity 39:383 (1983). The results are shown in Table 1. The results show that animals injected with SPE-A positive streptococci developed the

15   clinical features of STSS, and 6/8 succumbed. The two surviving animals developed antibodies to SPE-A. In contrast, the toxin negative strain, T253 cured, induced only fever, and no deaths were observed, even at much higher bacterial cell concentrations. As in the previous animal model experiments, no evidence of soft tissue necrosis was observed. Furthermore, the streptococci remained localized in

20   the golf balls, suggesting these streptococcal strains were not highly invasive.

Table 1: Induction of STSS by *speA* in a Wiffle ball Rabbit Model

Treatment	Average Highest Temperature (°C)	Dead/Total
None	39.1	0/4
T253 cured T12*	41.2	6/8[1]
T253 cured*	40.7	0/6
T253 cured+	41.0	0/6

*   Approximately 1 X 108 cells
+   Approximately 1 X 1011 cells
25   I   2 survivors developed antibodies to SPE-A

Immunization with recombinantly produced SPE-A decreased death rates when rabbits were challenged with M1 or M3 streptococci. Rabbits were

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immunized with cloned SPE-A derived from S. aureus to prevent the possibility of

immunizing the animals with contaminating streptococcal products, such as M

protein. Control animals were not immunized against SPE-A. The rabbits received

50 µg of recombinantly produced SPE-A in emulsified in Freund's incomplete

5   adjuvant subcutaneously. After 9 days, rabbits were challenged subcutaneously with

25 ml of M3 (1.4 X 109 total CFU) or M1 (4.2 X 109 total CFU) streptococci grown

in dialyzed beef heart medium. The M1 and M3 streptococcal isolates are clinical

isolates. The M1 isolate is designated MNST and the M3 isolate is designated

MNBY. These isolates are available from Dr. Schlievert, University of Minnesota,

10   Mpls. MN.

The data presented in Table 2 show the striking results of these experiments.

Table 2:   **Protection of Rabbits from STSS with necrotizing fascitis and**
myositis, induced by M3 or M1 streptococci, by prior
15   **immunization against SPE-A**

Number of Animals	Immunizing Agent[*]	Challenge Agent[+]	Number Alive[1] Total	
20	--	M3	4/20	
				P<<0.001'
20	SPE-A	M3	16/19	
17	--	M1	9/17	
				P<0.04'
15	SPE-A	M1	13/15	

*   Animals were immunized against cloned SPE-A prepared from S. aureus; ELISA titers against
SPE-A were greater than 10,000.
20 +   Animals were challenged subcutaneously with 1.4 X 109 CFU M3 or 4.2 X 109 CFU M1
streptococci in a dialyzable beef heart medium.
   1   According to the guidelines of the University of Minnesota Animal Care Committee, the
experiment which used M3 streptococci was terminated after 24h, and the experiment that used
M1 streptococci was terminated after 48h.
25   '   P values determined by Fisher's Exact Probability Test.

As indicated 16 of 19 SPE-A immunized rabbits survived challenge with M3

streptococci, whereas only 4 of 20 nonimmune animals survived. The surviving

30   immune animals showed clear evidence of contained soft abscess formation, upon

which examination of the fluid, was filled with PMNs. Similar results were obtained

in studies of M1 streptococci, except the M1 organisms were not as virulent as the

45

M3 organisms (Table 2). Higher numbers of M1 streptococci were used, and a
reduced death rate in the rabbits was seen, even in nonimmune control animals. This
may reflect the approximately 50-fold lower SPE-A production by M1 strains
compared to M3 strains.

5   In contrast, none of the nonimmune animals showed abscess formation, and
examination of fluid from 2/2 animals revealed no PMN infiltrate. These results
show that one major difference between the SPE-A immune versus nonimmune
animals appears to be whether or not an inflammatory response could be mounted.
Prior work showed that SPE-A, as well as other pyrogenic toxin superantigens,

10   induce macrophages to produce high levels of TNF-α. TNF-α greatly reduces PMN
chemotaxis, apparently through down regulation of chemotactic receptors.
Therefore, it is believed that the results show that antibodies in the SPE-A
immunized animals (titers > 10,000 by ELISA) block the release of TNF-α from
macrophages by neutralizing SPE-A, thus allowing the development of a protective

15   inflammatory response. In the nonimmune animals SPE-A could cause a significant
release of TNF-α which in turn prevents development of a protective chemotactic
response.

It is important to note that all of the animals that died except one showed
extensive soft tissue damage as evidenced by their entire sides turning purple-black

20   and in many cases sloughing. One animal in the immunized group died after
immunization. The lack of detectable inflammation in the tissue of these animals
suggest that streptococcal factors and not components of a host immune response
causes necrotizing fascitis and myositis. Other extracellular factors may also
contribute to the soft tissue damage, such as SPE B and streptolysins O and S.

25   All of the above data make a strong case for the causative role of pyrogenic
toxin superantigens, and particularly SPE-A, when present, in the development of
STSS.

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EXAMPLE 3
Site Directed Mutagenesis f a DNA Sequence Encoding SPE-A

Locations in the SPE-A molecule important for biological activity were

5   identified using site directed mutagenesis. Single amino acid changes were

introduced into various regions of the molecule as described below.

The model of the three dimensional structure of SPE-A is shown in Figure 1.

This model structure was constructed by Homology using an Insight/Homology

program from BioSym Corp., San Diego, CA. This molecule has several domains

10   identified as:

Domain	Corresponding Amino Acids
Helix 2	11-15
N terminal α-helix, helix 3	18-26
Domain B - β strands	
strand 1	30-36
strand 2	44-52
strand 3	55-62
strand 4	75-83
strand 5	95-106
Central α-helix, helix 5	142-158
Domain A - β strands	
strand 6	117-126
strand 7	129-135
strand 8	169-175
strand 9	180-186
strand 10	213-220
Helix 4	64-72
Helix 6	193-202

Amino acid number designations are made by reference to the sequence in

Figure 3.

Amino acids were selected in each of the domains and to alter the cysteine

15   residues in the molecule. The especially preferred regions are the N terminal a-helix

(18-26); the central a-helix (142 to 158); Domain A β strands and Domain B β

strands .

Target residues for mutagenesis were chosen among the conserved amino

acids throughout the pyrogenic toxin family by comparing primary amino acid

20   sequence and/or 3-D conformational similarities or homologies using computer

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programs as described previously. The changes made to each of the amino acids were selected to change the characteristics of the amino acid side chain of residue at the particular site. For example, at three of the residues (87, 90 and 98) serine was substituted for cysteine so as to alter the sulphydryl groups in the molecule. At three

5   other amino acid residues changes were made in the charge present at that site. For example, a lysine was changed to a glutamic (157) acid, lysine was changed to asparagine (16) and asparagine was changed to aspartic acid (20).

Other amino acids may affect the interaction of the toxins with MHC Class II molecules. In another molecule, the TSST-1 N terminal β barrel strands were

10   important for contacts with a and β chains of MHC class II molecules. Therefore, changes in the Domain A and Domain B β strands may be important for controlling the interaction of these molecules with MHC Class II molecules. In addition, changes in the residues can be prepared using random mutagenesis and substitution of each of the other 19 amino acids at a particular location, and then selecting those

15   mutants showing an alteration in biological activity such as lethality.

The mutant SPE-A molecules were prepared using site directed mutagenesis using polymerase chain reaction (PCR) in which the template DNA was the cloned SPE-A gene from phage T12. These primers were utilized for each mutation generated. Generation of each mutant involved using three primers as follows: an

20   upstream 5' flanking primer, an internal primer including the change in DNA sequence coding for a change in an amino acid and a downstream flanking primer. The upstream flanking primer was included in every PCR reaction and is homologous to a DNA region about 760 bases upstream of the translational start site and has a sequence:

25
```
5' GGT GGA TCC TTG AAA CAG GTG CA-3' (SEQ ID NO:11)
        BamH1
```

The resulting PCR product includes the *speA* promoter and possible upstream

30   regulatory region. The downstream flanking primer is complementary to a region of DNA about 270 bases downstream of the stop codon and has a sequence:

```
5' -CCC CCC GTC  GAC GAT AAA ATA GTT GCT AAG
                 Sal I
35    CTA CAA GCT-3'  (SEQ ID NO:2)
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The downstream flanking primer is present in every PCR reaction and because of the location of the primer the PCR product contains a putative transcription termination sequence.

Each mutation is generated using an internal primer including a DNA sequence coding for a change at a particular amino acid residue. The internal primers used to generate each mutant are as follows:

Mutant	Internal Primer
N20D	5' AAA AAC CTT CAA GAT ATA TAT TTT CTT -3' (SEQ ID NO:3)
C87S	5'-TCC-ACA-TAA-ATA GCT GAG ATG GTA ATA-TCC-3' (SEQ ID NO:4)
C90S	5'-CTC TGT TAT TTA TCT GAA AAT GCA GAA-3' (SEQ ID NO:5)
C98S	5' CCC TCC GTA GAT CGA TGC ACT CCT TTC TGC-3' (SEQ ID NO:6)
K157E	5'-CTT-ACA-GAT-AAT-GAG-CAA-CTA TAT ACT-3' (SEQ ID NO:7)
S195A	5'-CCA GGA TTT ACT CAA GCT AAA TAT CTT ATG-3' (SEQ ID NO:8)
K16N	5'- CAA CTT CAC AGA TCT AGT TTA GTT AAC AAC CTT-3' (SEQ ID NO:9) (forward primer) and
	5'- T TTG AAG GTT GTT AAC TAA ACT AGA TCT GTG AAG TTG-3' (SEQ ID NO:10) (backward primer)

The underlined residues indicate changes in coding sequence made from DNA sequence coding will type SPE-A.

PCR was conducted as follows: Briefly, a downstream flanking primer and a forward primer spanning the site of mutation and containing the nucleotide substitutions necessary to generate an amino acid change were mixed in unequal molarity in a standard PCR reaction. The DNA product obtained was prevalent in the strand containing the mutation. This product, or megaprimer, that can be several hundred bases long, was isolated by electrophoresis in 1% agarose gel and eluted by

the use of the Geneclean kit, as recommended by the manufacture (Bio 101, La Jolla, California).

Briefly, the PCR reaction conditions are: PCR is performed in a 100 ul reaction mixture containing 10 mM Tris-HCl (pH=8.3), 50 mM KCl, 1.5 mM

5   MgCl2, 200 uM each dNTP, 2 ng template plasmid DNA, 100 pmoles flanking primer, 5 pmoles internal primer, and 2.5 units of Ampli Taq DNA polymerase (Perkin Elmer Cetus). In the second amplification step, the composition of the reaction mix is as above except for equal molarity (5 pmoles each) of flanking primer and megaprimer and 1 ug template. PCR is conducted for 30 cycles of

10   denaturation at 94°C X 1 minute, annealing at 37°C or 44°C X 2minutes and elongation at 72°C for 3 minutes. Hybridization conditions can be varied in accord with known principles depending on the primer size, mismatches, and GC content.

A plasmid containing the *speA* cloned gene and flanking sequences was used as a template. In the second step, the megaprimer and an upstream flanking primer

15   were combined in the reaction mixture in equal molarity to generate the full length mutant *speA*.

The mutant *speA*s were digested with appropriate restriction enzymes and cloned into the shuttle vector pMIN 164 . This vector is a chimera of the E. coli plasmid pBR328, which carries an ampicillin resistance gene, and the staphylococcal

20   plasmid pE194, which confers erythromycin resistance. The ligated plasmid mixtures were transformed, selected for, and screened in E. coli. Clones positive for toxin production, as judged by double immunodiffusion assays, were sequenced by the method of Hsiao cited supra to confirm the presence of the desired mutation and the absence of other mutations. Plasmids were then transformed in S. aureus strain

25   RN 4220 (available from Richard Novick, Skirball Institute, New York, NY) for expression and production of mutant toxins.

S. aureus carrying the mutant or the wild type *speA*s in pMIN164 were grown at 37°C with aeration to stationary phase in dialyzable beef heart medium, containing 5μg/ml of erythromycin. Cultures were precipitated with four volumes

30   of ethanol and proteins resolubilized in pyrogen free water. The crude preparations were subjected to successive flat bed isoelectric focusing separations in pH gradients of 3.5 to 10 and 4 to 6. The fractions that were positive for toxin by antibody

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reactivity were extensively dialyzed against pyrogen free water, and an aliquot of
each was tested for purity by SDS polyacrylamide gel electrophoresis in 15%
(weight/volume) gels (data not shown). All mutants prepared were as resistant as
the native toxin to treatment for 60 minutes with trypsin (2 μg/μg SPE-A), and this

5   together with the conserved reactivity to polyclonal antibodies raised against native
SPE-A indicates that the mutations introduced do not cause gross structural changes
of the toxin. Using these methods, 7 mutants having single amino acid substitutions
in the amino acid sequence of SPE-A were generated.

10   **EXAMPLE 4**
                          Biological Activity Profile of Mutant SPE-A

        Biological activities of the mutant toxins were evaluated and compared to
those of the wild type SPE-A. The mutant toxins were tested for the ability to

15   stimulate proliferation of T lymphocytes (superantigenicity), to enhance host
susceptibility to endotoxin shock and for development of toxic shock syndrome and
lethality.

The ability to stimulate proliferation of T lymphocytes was measured as [3H]
thymidine incorporation into cellular DNA of rabbit splenocytes. A standard 4-day

20   mitogenicity assay was performed in 96 well microtiter plates. Each well contained
2 X 105 rabbit splenocytes resuspended in 200 μl RPMI 1640 (Gibco, Grand Island,
NY) supplemented with 25 mM HEPES, 2.0 mM L-glutamine, 100 U penicillin,
100 μg/ml streptomycin and 2% heat inactivated FCS. 20 μl samples of exotoxins
were added in quadruplicate amounts in final amounts: 1 μg to 10-5 μg/well. The

25   background cellular proliferation was determined in quadruplicate wells by adding
20 μl RPMI to the splenocytes. After 3 days of incubation in a humidified chamber
at 37°C and 7% CO2, 1.0 μCi (20 μl volume of 5-[methyl-3H]-thymidine (46
Ci/mmole, Amersham, Arlington Heights, IL) was added to each well and incubated
for 18 hours. Cellular DNA was collected on glass fiber filters and the [methyl-3H]

30   thymidine incorporation was quantified by liquid scintillation counting. Three
separate assays using three different rabbit donors were performed. Exoprotein
concentrations were tested in quadruplicate in each of three assays. Results are
presented as CPM.

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The ability to enhance host susceptibility to endotoxin shock was tested in American Dutch Belted rabbits. Animals weighing between 1 and 2 kg were injected in the marginal ear vein with 5 µg/kg body weight of SPE-A (equal to 1/50 LD50) and challenged 4 hours later by IV injection of 1 or 10 µg/kg body weight of

5   endotoxin (about 1/100 LD50) from Salmonella typhimurium. Control rabbits received injections with PBS. The animals were monitored after 48 hours for death.

Lethality was also measured using miniosmotic pumps implanted subcutaneously in American Dutch Belted rabbits and containing 200 µg of toxin. Individual proteins (200 µg) were injected in 0.2 ml PBS into miniosmotic pumps

10   (Alzet, AlzaCo, Palo Alto, CA). The pump is designed to deliver a constant amount of toxin over a 7-day period. Rabbits were monitored 3 times daily for signs of toxic shock syndrome such as diarrhea, erythema of conjunctivae and ears, shock and death for up to 8 days.

The results of the T cell mitogenicity studies are shown in Figures 4, 5 and 6.

15   The results show that the mutant N20D had a five-fold decrease in superantigenicity or T cell mitogenicity activity. Mutants C87S and C98S also had a 4-fold decrease in mitogenicity for T cells. Thus, several of the mutations affected biological activity of superantigenicity or T cell mitogenicity.

The results of enhancement of endotoxin shock and lethality are shown in

20   Tables 3, 4, and 5 shown below.

Table 3.   Mutants SPE-A-K16N and SPE-A-N20D assayed for ability to
                cause endotoxin enhancement or lethality when administered in
                subcutaneous miniosmotic pumps. Results are expressed as ratio
25   of deaths over total rabbits tested

	Protein		
	SPE-A	K16N	N20D
Endotoxin enhancement 1 µg/kg endotoxin)	3/3	6/7	0/3
Lethality in miniosmotic pumps	3/4	ND	0/4

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Table 4. Mutants SPE-A-C87S, SPE-A-C90S, and SPE-A-C98S tested for ability to induce endotoxin enhancement r lethality when administered in subcutaneous miniosmotic pumps. Results are expressed as ratio of deaths over total number of treated rabbits.

	Protein			
	SPE-A	C87S	C98S	C90S
Endotoxin enhancement 1 µg/kg body weight	2/3	1/3	0/3	ND
Endotoxin enhancement 10 µg/kg body weight	2/3	3/3	1/3	ND
Lethality in miniosmotic pumps	3/4	ND	ND	3/3

Table 5. Mutants SPE-A-K157E and SPE-A-S195A tested for ability to induce lethality when administered in subcutaneous miniosmotic pumps. Results are expressed as ratio of deaths over total number of treated rabbits

	Protein		
	SPE-A	K157E	S195A
Lethality in miniosmotic pumps	6/8	0/4	3/3

The results show that animals treated with the mutant N20D did not develop STSS when tested using either model of STSS. The mutation in N20D is located in an organized a-helix bordering the deep groove on the back of the toxin (Figure 1). This residue is important both in superantigenicity and lethality functions of the molecule.

Mutations that eliminated sulphydryl groups and, therefore, that interfere with possible disulfide linkages, have varied effects on the biological activities of SPE-A, depending on which cysteine residue was mutated. The C90S mutant remained completely lethal (Table 4), and T cell stimulatory activity was not significantly decreased (Fig. 5a). In contrast, C87S and C98S mutations reduced approximately four fold the toxin's mitogenicity (Fig. 5b). However, ability to cause endotoxin shock was affected differently by the two mutations, with C98S being only weakly toxic, but C87S being strongly toxic (Table 4). An explanation for these results is based upon the relative positions of the three cysteine residues in the primary sequence and in the 3-dimensional structure (Fig. 1). The lack of the sulfhydryl group of C98 may preclude formation of a putative disulfide bridge seen

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in staphylococcal enterotoxins, and therefore, the conformation of the loop would be lost. This would have detrimental effects for the activity if amino acids in this loop are responsible for contact with host cellular receptors or have some other function in biological activity of the molecule. In the case of C87S mutation, the putative

5   disulfide bond could still be created between C90 and C98, preserving most of the conformation and, therefore, the activity.

Mutant K157E, located within the long central a-helix, retained complete superantigenicity (Fig. 6b), but was nonlethal when administered in miniosmotic pumps to rabbits (Table 6).

10   Residue S195A, which is part of a-5 helix, may not be important for the biological activities tested, since its mutation does not affect activities tested thus far. This residue may not be exposed to the environment or may not contribute to binding.

These results show that lethality and superantigenicity can be affected by

15   mutations at several sites. Lethality can be affected by mutations in residues in the N terminal a-helix (N20D) and in the central a-helix (K157E). Mitogenicity can be affected by mutations in the N terminal a-helix and changes to sulfhydryl groups.

These results also show that mitogenicity and lethality are separable activities as mutants were generated that affect lethality without affecting

20   superantigenicity (K157E) and that affected mitogenicity without affecting lethality (C87S).

EXAMPLE 5

Preparation of Double or Triple Mutants of SPE-A using PCR

25   There are a number of methods that can be used to generate double or triple mutant SPE-A toxins or fragments thereof.

Mutant SPE-A toxins with two or more changes in amino acid sequences were prepared using PCR as described previously. In a first PCR reaction, an first internal primer coding for the first change at a selected site was combined with 5'

30   and 3' flanking primers to form a first PCR product. The first PCR product was a DNA sequence coding for a mutant SPE-A toxin having one change in amino acid sequence. This first PCR product then served as the template DNA to generate a

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second PCR product with two changes in amino acid sequence compared with a protein having wild type SPE-A activity. The first PCR product was the template DNA combined with a second internal primer coding for a change in amino acid at a second site. The second internal primer was also combined with the 5' and 3'

5   flanking primers to form a second PCR product. The second PCR product was a DNA sequence encoding a mutant SPE-A toxin with changes at two sites in the amino acid sequence. This second PCR product was then used as a template in a third reaction to form a product DNA sequence encoding a mutant SPE-A toxin with changes at three sites in the amino acid sequence. This method was utilized to

10   generate DNA sequences encoding mutant toxins having more than one change in the amino acid sequence.

An alternative method to prepare DNA sequences encoding more than one change is to prepare fragments of DNA sequence encoding the change or changes in amino acid sequence by automated synthesis. The fragments are then subcloned into

15   the wild type SPE-A coding sequence using several unique restriction sites. Restriction sites are known to those of skill of the art and are readily determined from the DNA sequence of a wild type SPE-A toxin. The cloning is done in a single step with a three fragment ligation method as described by Revi et al. Nucleic Acid Res. 16: 1030 (1988).

20   Mutant D45N was obtained by the *in vitro* site directed mutagenesis system Altered Sites II (Promega, Madison, WI). The 1.75 kb *Bam* HI-*Sal*I fragment of *speA* was subcloned in vector pAlter provided in the mutagenesis kit (Promega). The mutagenic oligonucleotide was CTT TTA TCT CAC AAT TTA ATA TAT
(SEQ ID NO: 11)
AAT G. The mutagenesis reactions were performed as suggested by the

25   manufacturer.

Generation of Triple Mutants

Single amino acid mutants, such as D45N described immediately above, were used to produce double mutants and the triple mutant by subcloning fragments

30   of *speA* carrying the desired new mutation into plasmids with single or double *speA* mutations. Table 10 describes the unique restriction sites used for the swapping of DNA segments and the recipient plasmid for each subcloning procedure. The

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mutants were sequenced in the region of the newly introduced mutation to confirm the subcloning was successful.

Table 10:   **List of multiple mutations introduced in SPE A and restriction fragments swapped to generate multiple mutants from single mutants.**

Mutant		Restriction Fragment	Donor	Recipient
N20D/D45N/C98S				
	Step 1	SalI-BstEII	D45N	N20D
	Step 2	BamHI-BfrI	N20D/D45N	C98S

Example 6

Toxicity Studies related to Single and Double Mutants

Wild type SPE A, SPE A N20D, SPE A K157E, SPE A N20/C98S, and SPE A N20D/K157E were evaluated for superantigenicity based on their capacity to stimulate rabbit splenocyte proliferation (see Figures 7 and 8).

Double mutants SPE A (N20D/C98S, N20D/K157E) were prepared by PCR mutagenesis using the techniques described above. The mutant SPE A gene, *speA* N20D, served as template DNA for introduction of the second mutation. The double mutant genes were sequenced as described above to insure that only the indicated changes were present. Only the desired changes were present.

Rabbit spleen cells were cultured in the presence of SPE A and SPE A mutants in vitro for 3 days and then an additional day after addition of 1μCi/well of 3H thymidine. Incorporation of 3H thymidine into lymphocyte DNA was used as the measure of T cell proliferation. A superantigenicity index was calculated as average counts/min 3H thymidine incorporation in stimulated cells divided by average counts/min in cells cultured without added SPE A or mutants.

Wild type SPE A was significantly superantigenic at doses from 1 to 0.001 μg/well (Figure 7). SPE A K157E was significantly mitogenic at doses of 0.01 and 0.001 μg/well (Figure 7). The three other SPE A mutants (SPE A N20D, SPE A N20D/C98S, SPE A N20D/K157E) were significantly less superantigenic (Figure 8) than wild type SPE A at doses of 1 to 0.001 μg (p<0.001). Interestingly, SPE A

S_b

N20D was significantly more superantigenic (Figure 8) than SPE A N20D/C98S at doses of 1 and 0.1 μg (p<0.0005, p<0.001, respectively). Furthermore, SPE A N20D was more mitogenic than SPE A N20D/K157E at the 1 μg/well dose (p<0.01). Thus, the data indicated the N20D/C98S mutant had less toxicity than the single N20D mutant, and the double mutant N20D/K157E was intermediate between the other two proteins. All three mutants were significantly less toxic than wild type SPE A.

In a second experiment rabbits (3/group) were challenged iv with 10 μg/kg SPE A or mutants and then endotoxin 5 μg/kg) 4 hours later. Animals were monitored for 48 hours for enhanced lethality due to administration of SPE and endotoxin. This assay is the most sensitive in vivo measure of SPE A lethal activity. As indicated in Table 6, 0/3 animals challenged with wild type SPE A and endotoxin survived. In contrast all but one animal challenged with SPE A N20D survived, and all animals challenged with SPE A N20D/C98S or SPE A N20D/K157E survived.

Table 6:   **Capacity of SPE A (10 μg/kg) or mutants (10μg/kg) to enhance rabbit susceptibility to the lethal effects of endotoxin (5 μg/kg)**

SPE A or Mutant	Number Dead/Total
Wild type SPE A	3/3
SPE A N20D	1/3
SPE A N20D/C98S	0/3
SPE A N20D/K157E	0/3

Note: SPE A or mutants were administered iv at 0 hour and endotoxin iv at 4 hours. Animals were monitored for 48 hours for lethality.

In a third experiment rabbits were immunized with SPE A N20D, SPE A N20D/C98S, OR SPE A N20D/K157E, and then challenged with wild type SPE A (10 μg/kg) and endotoxin (5 μg/kg or 25 μg/kg) as in the preceding experiment. Control animals were not immunized but were challenged with wild type SPE A plus endotoxin. Rabbits were immunized every other week for two injections, with mutant proteins (50 μg/injection) emulsified in incomplete adjuvant (Freunds, Sigma Chemical Co., St. Louis, MO) and then rested one week prior to challenge with wild type toxin. The combination of wild type SPE A and endotoxin represent 20 LD50

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for challenge with 10 μg/kg SPE A and 5 μg/kg endotoxin, and 100 LD50 for

challenge with 10 μg/kg SPE A and 25 μg/kg endotoxin.

As indicated in Table 7, all animals challenged with 100 LD50 of SPE A and

endotoxin succumbed. Similarly, all animals immunized with SPE A N20D or

5   N20D/K157E succumbed when challenged with 20 LD50 of SPE A and endotoxin.

In contrast, animals immunized with the double mutant N20D/C98S survived.

Animals immunized with the double mutant N20D/K157E succumbed earlier than

other animals. The data above indicates that double mutants and in particular SPE A

N20D/C98S shows effectiveness as a toxoid vaccine in test animals.

10

Table 7:   **Ability of SPE A mutants to immunize rabbits against the capacity of wild type SPE A to enhance susceptibility to lethal endotoxin shock.**

Immunizing Agent	Challenge dose of SPE A and Endotoxin	Number Dead/Total
None	10 μg/kg SPE A, 25 μg/kg endotoxin	3/3
SPE A N20D	10 μg/kg SPE A, 25 μg/kg endotoxin	2/2
SPE A N20D/C98S	10 μg/kg SPE A, 25 μg/kg endotoxin	2/2
SPE A N20D/K157E	10 μg/kg SPE A, 25 μg/kg endotoxin	2/2
None	10 μg/kg SPE A, 5 μg/kg endotoxin	3/3
SPE A N20D	10 μg/kg SPE A, 5 μg/kg endotoxin	2/2
SPE A N20D/C98S	10 μg/kg SPE A, 5 μg/kg endotoxin	0/3
SPE A N20D/K157E	10 μg/kg SPE A, 5 μg/kg endotoxin	3/3

15

Note: Some animals escaped during this experiment. These animals were not included in
the above data.

Example 7
SPE A Inhibition by Antibodies to SPE-A Mutants
20   **and SPE-A mutant immunization**

One ml of blood was drawn from the marginal ear vein from each of the

rabbits immunized with N20D, N20D/C98S, and N20D/K157E SPE A and

nonimmunized controls. Animals were bled 6 days after the last immunization (one

25   day before animals were used in the experiment in Table 6). After the blood clotted,

sera were separated by centrifugation (13,000xg, 10 min). Sera from each group

were pooled and treated with 33 1/3% (final concentration) of ammonium sulfate for

1hr at room temperature to precipitate immunoglobulins. Precipitated

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immunoglobulins were collected by centrifugation (13,000 xg, 10 min),

resolubilized to the original volume in phosphate-buffered saline (0.005M NaPO4

pH7.0, 0.15M NaCl), and dialyzed for 24 hr against 1 liter of 0.15M NaCl at 4°C.

The dialysates were filter sterilized (0.45μm pore size) and used in studies to

5   neutralize rabbit splenocyte mitogenicity (superantigenicity) of 0.01μg SPE A

(Figure 9). Serum from one rabbit immunized with sublethal doses of wild type SPE

A was fractionated comparably and used as the positive control. Twenty microliters

of the immunoglobulin fractions (Igs) from each group of sera were diluted 1/5 and

1/50 with complete RPMI 1640 mammalian cell culture media (dilution with respect

10   to the original serum volume) and added to each of 4 wells containing wild type SPE

A and 2 X 105 rabbit splenocytes in our standard mitogenicity assay. Igs and wild

type toxin were both added to lymphocytes at time 0. The results are shown in

Figure 9.

The 1/5 diluted Igs, whether from immunized animals or nonimmune

15   controls were inhibitory to splenocyte proliferation, probably because of residual

ammonium sulfate in the Igs. However, Igs from the SPE A immune animals and

Igs from N20D, N20D/C98S, and N20D/K157E immune animals were more

inhibitory than Igs from nonimmune controls (p=0.006 for SPE A versus

nonimmune, [=0.035 for N20D versus nonimmune, p=0.0002 for N20D/C98S

20   versus nonimmune, and p=0.0001 for N20D/K157E versus nonimmune by use of

Student's t test analysis of normally distributed unpaired data), indicating specific

inhibition of mitogenicity.

When Igs were added at the 1/50 dilution, the double mutant N20D/C98S

caused significant inhibition of splenocyte proliferation compared to nonimmune

25   controls (p=0.046). At this Ig concentration none of the fractions caused nonspecific

suppression of lymphocyte mitogenicity.

These data suggest that the double mutant N20D/C98S was better able to

immunize animals against mitogenicity of the wild type SPE A than the single

mutant N20D or the other double mutant N20D/K157E. However, the double

30   mutant N20D/K157E was a better immunogen than the single mutant N20D.

Without being bound by the following, it is possible the two changes in the

N20D/C98S mutant interfere with host cell receptor sites required for lethality, T

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cell receptor interaction, and possibly indirectly, class II MHC interaction on antigen

presenting cells. Since class II MHC interaction depends on amino acid residues in

the β barrel domain (domain B) in the standard view of the toxin, we propose also

that a change in this region (such as D45N) may improve the immunogenicity of

5   N20D/C98S even more. The basis for this hypothesis is that wild type toxin (and

possibly mutants lacking changes in the class II MHC interaction domain) bind

directly to class II MHC molecules without the requirement for normal processing

by antigen presenting cells. Mutants that contain amino acid changes that interfere

with this direct class II MHC interaction may be more immunogenic since the

10   mutants maybe more easily internalized and processed. Thus, the triple mutant

N20D/C98S/D45N will be evaluated using the methods used to evaluate the other

mutants.

          Sera obtained from the nonimmune controls and each group of N20D,

N20D/C98S, or N20D/K157E immunized rabbits were tested directly for ELISA

15   titer against wild type SPE A (L. Hudson and F.C. Hay, Practical Immunology 2nd

Ed, 1980, Blackwell Scientific Publications, Boston p 237-239.) Serum from each

animal was evaluated separately. The antibody titers obtained were averaged and

are shown in Table 8. Nonimmune control animals as expected had very low titers

of antibodies against SPE A. In contrast all animals immunized against the mutants

20   had significant antibody titers. The animals immunized with the double mutant

N20D/K157E had the highest average titer with the other two mutants being

comparable. However, the range of titers for the N20D immunized animals was

much greater (20, 40, 160, 640, 640 for each of the 6 animals) than either of the

double mutants. The data suggest the double mutants gave more consistent

25   immunization.

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Table 8: ELISA antibody titers of animals immunized against N20D, N20D/C98S, N20D/K157E SPE A and nonimmune controls

Immunizing Agent	Average Antibody Titera	Rangeb
None	10	<10-20
N20D SPE A	250	20-640
N20D/C98S SPE A	80	80
N20D/K157E SPE A	425	320-640

5   a   6 animals/group

      b   The lowest titer detectable was 10. Titer is the reciprocal of the last dilution that gave a positive result.

In a final experiment animals (3/group) were immunized against N20D,

10   N20D/C98S, or N20D/K157E (50μg/injection iv) by administering mutant protein every other day for 5 injections and then resting the animals for one day. Animals were then evaluated for immunity against the ability of wild type SPE A to cause fever [20 times the minimum pyrogenic dose (MPD) 4 hours after injection/kg body weight (20 MPD-4)]. SPE A is one of the most potent pyrogens known with one

15   MPD-4 in rabbits of 0.15 μg/kg. At the 4 hr timepoint animals were injected with endotoxin (25 μg/kg) to evaluate immunity to the enhanced susceptibility to endotoxin shock. The results are shown in Table 9.

The nonimmune animals and those immunized with N20D SPE A showed both significant fever responses (0.8 °C for both groups) and enhanced susceptibility

20   to endotoxin (2/3 succumbed in 48 hr in both groups). In contrast animals immunized with either double mutant were completely protected from fever and the enhancement phenomenon.

Collectively, all of the above data suggest both double mutants are better able to immunize animals against the toxic effects of SPE A than the single mutant.

25   None of the mutants themselves were toxic to the animals. The double mutant N20D/C98S was a better immunogen than N20D/K157E, but both were effective.

Table 9:   Ability of SPE A mutants N20D, N20D/C98S, and N20D/K157E
to immunize rabbits against SPE A pyrogenicity and lethal
challenge by SPE A and endotoxin.

Immunizing Agent	Fever Response Change °C at 4 hr	Number Dead/Total
None	0.8	2/3
N20D SPE A	0.8	2/3
N20D/C98S SPE A	0.0	0/3
N20D/K157E SPE A	0.1	0/3

Example 8
Evaluation and Properties of Triple Mutant

Construction of triple mutant and stability determination.

Mutant N20D/D45N/C98S was constructed by two rounds of subcloning.
The resulting mutant *speA* was sequenced to ensure that in the cloning process the
DNA fragments containing the appropriate mutations were ligated. Plasmid
pMIN164 carrying the triple mutant *speA* gene was transformed in *Staphylococcus
aureus* RN4220, and triple mutant protein was produced and purified as described
for the other mutants.

Protein N20D/D45N/C98S was evaluated for stability. The protein was
purified from bacterial cultures in amounts comparable to the double mutant SPE.
The triple mutant protein also reacted with polyclonal antibodies specific for wild
type SPE A in double immunodiffusion assays. Moreover, N20D/D45N/C98S was
resistant to trypsin cleavage equally to wild type.

Proliferative activity of triple mutant protein.

Mutant N20D/D45N/C98S was evaluated for its proliferative activity in
rabbit and murine splenocytes and human PBMCs. The protein was much less
active than wild type SPE A in inducing rabbit (Table 11) and human (Table 13) cell
proliferation. In murine cells the protein activity was close to 50% the wild type's at
100 ng/well, and even higher when the 1,000 ng/well toxin dose was used (Table
12). In rabbit cells N20D/D45N/C98S was also less active than the single mutants
tested, N20D and D45N, and as active as the double mutant N20D/C98S (Table 11).
However, in the murine system, the triple mutant induced cell proliferation equally
to the single mutant protein D45N and was more active than N20D/C98S (Table 12),

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whereas in human cells N20D/D45N/C98S was also as active as D45N at 100 ng/well, but was much less active at 10 ng/well (Table 13). It appeared that the introduction of the third mutation increased the protein activity. Perhaps, the loss of charge of the Asp to Asn change at position 45 had a stabilizing effect on the protein lacking the sulfhydryl group of Cys 98.

Table 11:   Proliferative ability of triple mutant SPE A for rabbit splenocytes, compared to single mutants, double mutant and wild type SPE A.

Protein	100 ng/well			10 ng/well			1 ng/well		
	cpm $(10^3)^a$	SD^b	$\%^c$	cpm (10^3)	SD	%	cpm (10^3)	SD	%
SPE A	97	8		116	20		99	7	
N20D	23	4	23	9	2	8	3	1.4	3
D45N	10	1.3	10	21	4.5	18	3.2	1.7	3
N20D/C98S	13	2	13	2.4	1.5	2	0.5	1.5	0.5
N20D/D45N/C98S	7.6	2	8	4.4	1.3	4	1.7	0.4	2

a   Resulting from incorporation of [^{3}H]thymidine into DNA of proliferating splenocytes.
b   For quadruplicate samples.
c   Mutant activity divided by wild-type SPE A activity at the same dose and in the same assay x 100.

Table 12:   Proliferative ability of triple mutant SPE A on murine splenocytes, compared to single mutants, double mutant and wild type SPE A.

Protein	1,000 ng/well			100 ng/well		
	cpm $(10^3)^a$	SD^b	$\%^c$	cpm (10^3)	SD	%
SPE A	23	2.5		NT^d		
N20D/C98S	0.6	0.5	2.6	NT		
SPE A	15	3.4		52	1.8	
D45N	9	2.4	60	24	8.4	44
N20D/D45N/C98S	12	5	80	23	1.8	46

a   Resulting from incorporation of [^{3}H]thymidine into DNA of proliferating splenocytes.
b   For quadruplicate samples.
c   Mutant activity divided by wild-type SPE A activity at the same dose and in the same assay x 100.
d   NT, not tested.

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Table 13: **Proliferative ability of triple mutant SPE A on human PBMCs, compared to single mutant, double mutant and wild type SPE A.**

Protein	100 ng/well			10 ng/well		
	cpm $(10^3)^a$	SD[b]	%[c]	cpm (10^3)	SD	%
SPE A	31	3		22	2	
D45N	11	1.5	35	14.4	1.5	65
N20D/C98S	2.6	1	8	0.1	0.3	0.5
N20D/D45N/C98S	10	0.6	32	0.9	0.3	4

a   Resulting from incorporation of [^{3}H]thymidine into DNA of proliferating splenocytes.
b   For quadruplicate samples.
c   Mutant activity divided by wild-type SPE A activity at the same dose and in the same assay x 100.
d   NT, not tested.

Induction of IFN-γ and TNF-α secretion.

The N20D/D45N/C98S protein was also evaluated for its ability to induce secretion of IFN-γ and TNF-α from murine splenocytes and human PBMCs. These cytokines were measured in the supernates of cell cultures used to test proliferation (Tables 12 and 13). Supernates for cytokine determination were recovered after 96 hours of incubation from the cell cultures treated with the mutant or wild type SPE As at the doses of 1,000 ng/well and 100 ng/well for murine and human cells, respectively. Secretion of both IFN-γ and TNF-α was affected more in human than murine cells (Table 14). This appeared to correlate with the levels of cell proliferation observed (Tables 12 and 13). However, within each species, TNF-α secretion appeared less dependent on cell proliferation (Table 14). Human cells, which proliferated equally upon stimulation with D45N or the triple mutant protein, secreted greater amounts of TNF-α when treated with N20D/D45N/C98S. On the contrary, murine cells, which proliferated better when treated with N20D/D45N/C98S, secreted smaller amounts of TNF-α upon stimulation with the same toxin. Very little secretion of cytokines was observed in supernates of cells treated with hyaluronidase, and of untreated human cells, but murine untreated control cells were considerably active in secretion of both cytokines tested. This may in part contribute to the surprisingly high levels of IFN-γ and TNF-α in supernates of murine cells treated with either mutant protein tested (Table 14).

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Table 14.   Induction of cytokine secretion by triple mutant SEC A in murine splen cytes and human PBMCs

Protein	IFN-γ[a]				TNF-ά[b]			
	Human[c]		Murine[d]		Human		Murine	
	pg/ml[e]	%	pg/ml	%	pg/ml	%	pg/ml	%
N20D/D45N/C98S	2880	27	2508	41	782	59	680	72
D45N	2708	26	3058	50	428	32	898	96
SPE A	10484		6076		1336		940	
Hyaluronidase	0		0		46		11	
None	0		592	2	40		305	

a   IFN-γ, interferon -γ
b   TNF-ά, tumor necrosis factor-ά
c   Human PBMCs, 2x10⁵/well, were stimulated with 100 ng/well of wild type or mutant toxins. Samples for each proliferation assay were harvested at 96 hours and cytokine concentrations in the supernates were determined.
d   Murine splenocytes, 5x10⁵/well, were stimulated with 1,000 ng/well of wild type or mutant toxins. Samples for each proliferation assay were harvested at 96 hours and cytokine concentrations in the supernates were determined.
e   pg/ml of cytokine released upon mutant stimulation divided by pg/ml released upon wild type stimulation, in the same assay

Toxicity of N20D/D45N/C98S protein. Protein N20D/D45N/C98S was

assayed for its activity in enhancing endotoxin shock in American Dutch belted

rabbits. Young adult animals were injected i.v. with 5 μg/kg of body weight of

N20D/D45N/C98S or wild type SPE A proteins. Four hours later animals were

administered i.v. 10 μg/kg of body weight of endotoxin from *Salmonella*

typhimurium. Animals were monitored for symptoms of STSS and death for the 48

hours after the injection of endotoxin. Results are shown in Table 15. All animals

administered the N20D/D45N/C98S protein survived and their necroscopic

examination revealed no organ damage. On the contrary, all animals treated with

wild type SPE A died. This result indicated that the triple mutant toxin has no

detectable toxicity *in vivo.*

**Table 15.   Lethality and toxicity of triple mutant N20D/D45N/C98S SPE A
in the rabbit endotoxin enhancement model**

Protein	Multiorgan toxicity[a]	No dead/total animals[b]	p[c]
N20D/D45N/C98S	0/9	0/3	0.05
SPE A	ND[d]	3/3	

a   As judged by necroscopic examination of liver, spleen, lungs, and heart of surviving animals
only. Each damaged organ of every animal is given one point. The sum of possible points
is 3/animal. Numbers refer to total damage-points/group of animals.

b   Animals were administered intravenously 5 µg/kg of body weight of SPE A wild type or
mutant. Four hours later they were administered 10 µg/kg of body weight of endotoxin
from *Salmonella typhimurium*.

c   Comparison of lethality caused by SPE A triple mutants with lethality of wild type.

d   ND, not determined.

Antigenicity of D45N and N20D/D45N/C98S proteins. The proteins D45N,

N20D/C98/S, N20D/D45N/C98S and the starting mutant protein N20D were

evaluated for their abilities to stimulate in animals an antibody response specific for

wild type SPE A. Five groups of 5 American Dutch belted rabbits each were either

untreated or treated with one of D45N, N20D, N20D/C98S, N20D/D45N/C98S.

Proteins were administered subcutaneously in 25 µg doses for three times in IFA,

every other week. Titers of anti-SPE A antibodies were determined by ELISA in

sera obtained seven days after the last immunization. As shown in Table 16, animals

in all but the untreated group had antibody titers significantly higher than the

corresponding pre-immune titers. Moreover, all

Table 16. Antigenicity of purified triple mutant SPE A in rabbits compared to single mutants, double mutant, and wild type SPE A

Immunizing agent	Pre-immune[a]		Immune[c]		p[d]
	titer[b]	range	titer	range	
None	18	10-20	24	20-40	0.21
N20D	60	20-160	1600	1000-2000	0.003
D45N	26	10-40	6400	4000-8000	0.003
N20D/C98S	32	20-80	1220	100-2000	0.03
N20D/D45N/C98S	16	10-40	3400	1000-8000	0.037

5   a   Rabbits were bled prior to administration of the first toxin dose.
         b   Average titer of sera from five rabbits
         c   Rabbits were bled seven days after the administration of the third immunizing dose.
         d   Comparison of pre-immune serum titer within each group with the titer after immunizations
               by two-tailed t-test, assuming unequal variances

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Table 17.   **Significance in titer differences of sera from groups of animals immunized with different agents, determined by two-tailed t-test, assuming unequal variances**

Immunizing Agent	None	N20D/D45N/C98S	D45N	N20D/C98S
N20D	<0.001	0.0656	<0.001	0.3471
N20D/C98S	0.0015	0.0748	0.0185	
D45N	<0.001	0.0656		
N20D/D45N/C98S	<0.001			

15

immunized groups had antibody titers significantly higher than the non-immune

control group (Table 17). Protein D45N was the most immunogenic, stimulating an

average titer of 6,400, and with a range of only two serial 1:2 dilutions (Table 16).

This protein was significantly more effective as an antigen than N20D (Table 17).

20   When D45N was present in the same molecule as N20D and C98S its immunogenic

ability decreased considerably, as indicated by the average titer of 3,400 (Table 17).

The consistency of the antibody response to N20D/D45N/C98S was also less

compared to N20D alone, with titers ranging between 1000 and 8000 (4 1:2

dilutions). However, by comparing the log of the D45N-immune and

25   N20D/D45N/C98S-immune titers by use of the t-test, the two groups can be

considered different only with 10% confidence (Table 17). Similarly, titers from

N20D-immune and N20D/C98S-immune rabbits (1,600 and 1,220 respectively), that

were not significantly different from each other (Table 26 and 27), each showed a

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10% confidence difference to the N20D/D45N/C98S-immune titers. In conclusion, the N20D/D45N/C98S protein had an intermediate ability to elicit an antibody response to SPE A.

Protective ability of N20D/D45N/C98S. The triple mutant protein was evaluated and compared to N20D, D45N and double mutant N20D/C98S for its ability to protect animals from challenge with the wild type SPE A. Rabbits from the previous section, whose antibody titers are shown in Table 16, were challenged by use of the miniosmotic pump model. Pumps were loaded with 500 µg (equal to 2.5 times the lethal dose) of SPE A obtained from *S. pyogenes*. Animals were monitored for symptoms of STSS and death for 15 days after implantation of the miniosmotic pumps. Rectal temperatures were taken once before, and once two days after implantation. All animals immunized with one of the SPE A toxoids survived the challenge, whereas all five animals of the non-immune group died (Table 18).

Table 18. Immunizing ability of double and triple mutant SPE A compared to single mutants

Immunizing agents	Multiorgan toxicity[a]	No. with fever/total animals[b]	No. dead/total animals[c]	p[d]
None	17/20	4/5	5/5	
N20D	0/15	0/5	0/5	0.004
N20D/C98S	0/15	0/5	0/5	0.004
D45N	0/15	2/5	0/5	0.004
N20D/D45N/C98S	0/15	1/5	0/5	0.004

a   As judge by necroscopic examination of liver, spleen, lungs, and heart. Each damaged organ of each animal is given one point. The sum of possible points is 20 for the control group, and 15 for the treated groups (lungs were omitted). Fractions refer to total damage-points/total point per group of animals.

b   In degrees Celsius. Rectal temperatures were taken at baseline and at day 2 after implantation of miniosmotic pumps. Fever was considered as any temperature increment $\geq$ 0.5°C.

c   Miniosmotic pumps were loaded with 500 µg of wild type SPE A.

d   Comparison of lethality data of the vaccinated group of animals versus the untreated group by Fisher's exact probability test.

Lethality results were significant (p=0.004). Four animals of the non-immune group had a significant increase (more than 0.5°C) in body temperature (Table 18). Of the immunized groups, the D45N- and N20D/D45N/C98S-immune had some animals

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developing fever (2/5 and 1/5 respectively). All animals were evaluated for gross

organ abnormalities either after death (controls) or after being euthanized (treated).

None of the immunized animals had any organ damage (Table 18). This indicated

that the vaccination did not have toxic effects on the rabbit and that the antibodies to

5   the toxoids in all animals were able to block toxicity of the challenging wild type

SPE A. Conversely, the non-immune animals had 17 organ damage-points out of

the possible 20 (Table 18), indicating that each rabbit had at least two abnormal-

looking organs. These results together indicated that the vaccination with the

N20D/D45N/C98S mutant was safe and effective in protecting animals in an STSS

10   model.

Although the invention has been described in the context of particular

embodiments, it is intended that the scope of coverage of the patent not be limited to

those particular embodiments, but is determined by reference to the following

claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANT:
        (A) NAME: Regents of the University of Minnesota
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        (C) CITY: Minneapolis
        (D) STATE: Minnesota
        (E) COUNTRY: United States of America
        (F) POSTAL CODE (ZIP): 55415-1226

    (ii) TITLE OF INVENTION: MUTANTS OF STREPTOCOCCAL TOXIN A AND
        METHODS OF USE

   (iii) NUMBER OF SEQUENCES: 13

    (iv) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

    (v) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE: 07-JUN-1996
        (C) CLASSIFICATION:

    (vi) PRIOR APPLICATION DATA:
        (A) APPLICATION NUMBER: US 08/480,261
        (B) FILING DATE: 07-JUN-1995
        (C) CLASSIFICATION:

70

(2) INFORMATION FOR SEQ ID NO:1:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 29 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCATCACGGG TGGATTCTTG AAACAGGTG
    29

(2) INFORMATION FOR SEQ ID NO:2:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 47 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCATCACGCC CCCCGTCGAC GATAAAATAG TTGCTAAGCT ACAAGCT
    47

(2) INFORMATION FOR SEQ ID NO:3:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 172 base pairs
             (B) TYPE: nucleic acid
             (C) STRANDEDNESS: single
             (D) TOPOLOGY: linear

       (ii) MOLECULE TYPE: DNA (genomic)

       (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CCATCACCAT CACCAAGAAG AAATAATTAC ATATTAAATA CAATACATAT GTAATAATAA
    60

TAAATATATA AATAAAATAA TTACATATTA AAAATAATAC TTAATTATAA AAACACTATA
    120

ATTTCCATAA ATATTAATAA ATAATTAAAA ATAAAATAAT AAATAATTAA TC
    172

71

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 172 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCATCACCAT CACCAAGAAG AAATAATTAC ATATTAAATA CAATACATAT GTAATAATAA
   60

TAAATATATA AATAAAATAA TTACATATTA AAAATAATAC TTAATTATAA AAACACTATA
   120

ATTTCCATAA ATATTAATAA ATAATTAAAA ATAAAATAAT AAATAATTAA TC
   172

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 172 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CCATCACCAT CACCAAGAAG AAATAATTAC ATATTAAATA CAATACATAT GTAATAATAA
   60

TAAATATATA AATAAAATAA TTACATATTA AAAATAATAC TTAATTATAA AAACACTATA
   120

ATTTCCATAA ATATTAATAA ATAATTAAAA ATAAAATAAT AAATAATTAA TC
   172

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 172 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCATCACCAT CACCAAGAAG AAATAATTAC ATATTAAATA CAATACATAT GTAATAATAA
60

TAAATATATA AATAAAATAA TTACATATTA AAAATAATAC TTAATTATAA AAACACTATA
120

ATTTCCATAA ATATTAATAA ATAATTAAAA ATAAAATAAT AAATAATTAA TC
172

(2) INFORMATION FOR SEQ ID NO:7:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 172 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCATCACCAT CACCAAGAAG AAATAATTAC ATATTAAATA CAATACATAT GTAATAATAA
60

TAAATATATA AATAAAATAA TTACATATTA AAAATAATAC TTAATTATAA AAACACTATA
120

ATTTCCATAA ATATTAATAA ATAATTAAAA ATAAAATAAT AAATAATTAA TC
172

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 172 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CCATCACCAT CACCAAGAAG AAATAATTAC ATATTAAATA CAATACATAT GTAATAATAA
60

TAAATATATA AATAAAATAA TTACATATTA AAAATAATAC TTAATTATAA AAACACTATA
120

ATTTCCATAA ATATTAATAA ATAATTAAAA ATAAAATAAT AAATAATTAA TC
172

73

(2) INFORMATION FOR SEQ ID NO:9: -

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 172 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CCATCACCAT CACCAAGAAG AAATAATTAC ATATTAAATA CAATACATAT GTAATAATAA
    60

TAAATATATA AATAAAATAA TTACATATTA AAAATAATAC TTAATTATAA AAACACTATA
    120

ATTTCCATAA ATATTAATAA ATAATTAAAA ATAAAATAAT AAATAATTAA TC
    172

(2) INFORMATION FOR SEQ ID NO:10:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 172 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CCATCACCAT CACCAAGAAG AAATAATTAC ATATTAAATA CAATACATAT GTAATAATAA
    60

TAAATATATA AATAAAATAA TTACATATTA AAAATAATAC TTAATTATAA AAACACTATA
    120

ATTTCCATAA ATATTAATAA ATAATTAAAA ATAAAATAAT AAATAATTAA TC
    172

(2) INFORMATION FOR SEQ ID NO:11:

        (i) SEQUENCE CHARACTERISTICS:
                (A) LENGTH: 31 base pairs
                (B) TYPE: nucleic acid
                (C) STRANDEDNESS: single
                (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCATCACGGG TGGATCCTTG AAACAGGTGC A
    31

(2) INFORMATION FOR SEQ ID NO:12: -   74

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1851 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (ix) FEATURE:
        (A) NAME/KEY: CDS
        (B) LOCATION: 828..1583

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

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CCATCACGCA TCACTCATGT TTGACAGCTT ATCATCGATA AGCTTACTTT TCGAATCAGG
    60

TCTATCCTTG AAACAGGTGC AACATAGATT AGGGCATGGA GATTTACCAG ACAACTATGA
   120

ACGTATATAC TCACATCACG CAATCGGCAA TTGATGACAT TGGAACTAAA TTCAATCAAT
   180

TTGTTACTAA CAAGCAACTA GATTGACAAC TAATTCTCAA CAAACGTTAA TTTAACAACA
   240

TTCAAGTAAC TCCCACCAGC TCCATCAATG CTTACCGTAA GTAATCATAA CTTACTAAAA
   300

CCTTGTTACA TCAAGGTTTT TTCTTTTTGT CTTGTTCATG AGTTACCATA ACTTTCTATA
   360

TTATTGACAA CTAAATTGAC AACTCTTCAA TTATTTTTCT GTCTACTCAA AGTTTTCTTC
   420

ATTTGATATA GTCTAATTCC ACCATCACTT CTTCCACTCT CTCTACCGTC ACAACTTCAT
   480

CATCTCTCAC TTTTTCGTGT GGTAACACAT AATCAAATAT CTTTCCGTTT TTACGCACTA
   540

TCGCTACTGT GTCACCTAAA ATATACCCCT TATCAATCGC TTCTTTAAAC TCATCTATAT
   600

ATAACATATT TCATCCTCCT ACCTATCTAT TCGTAAAAAG ATAAAAATAA CTATTGTTTT
   660

TTTTGTTATT TTATAATAAA ATTATTAATA TAAGTTAATG TTTTTTAAAA ATATACAATT
   720

TTATTCTATT TATAGTTAGC TATTTTTTCA TTGTTAGTAA TATTGGTGAA TTGTAATAAC
   780

CTTTTTAAAT CTAGAGGAGA ACCCAGATAT AAAATGGAGG AATATTA ATG GAA AAC
   836
                                                    Met Glu Asn
                                                      1

AAT AAA AAA GTA TTG AAG AAA ATG GTA TTT TTT GTT TTA GTG ACA TTT
   884
Asn Lys Lys Val Leu Lys Lys Met Val Phe Phe Val Leu Val Thr Phe
      5                  10                  15
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- 75

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CTT GGA CTA ACA ATC TCG CAA GAG GTA TTT GCT CAA CAA GAC CCC GAT
    932
Leu Gly Leu Thr Ile Ser Gln Glu Val Phe Ala Gln Gln Asp Pro Asp
    20              25              30              35

CCA AGC CAA CTT CAC AGA TCT AGT TTA GTT AAA AAC CTT CAA AAT ATA
    980
Pro Ser Gln Leu His Arg Ser Ser Leu Val Lys Asn Leu Gln Asn Ile
              40              45              50

TAT TTT CTT TAT GAG GGT GAC CCT GTT ACT CAC GAG AAT GTG AAA TCT
    1028
Tyr Phe Leu Tyr Glu Gly Asp Pro Val Thr His Glu Asn Val Lys Ser
              55              60              65

GTT GAT CAA CTT TTA TCT CAC CAT TTA ATA TAT AAT GTT TCA GGG CCA
    1076
Val Asp Gln Leu Leu Ser His His Leu Ile Tyr Asn Val Ser Gly Pro
            70              75              80

AAT TAT GAT AAA TTA AAA ACT GAA CTT AAG AAC CAA GAG ATG GCA ACT
    1124
Asn Tyr Asp Lys Leu Lys Thr Glu Leu Lys Asn Gln Glu Met Ala Thr
            85              90              95

TTA TTT AAG GAT AAA AAC GTT GAT ATT TAT GGT GTA GAA TAT TAC CAT
    1172
Leu Phe Lys Asp Lys Asn Val Asp Ile Tyr Gly Val Glu Tyr Tyr His
100             105             110             115

CTC TGT TAT TTA TGT GAA AAT GCA GAA AGG AGT GCA TGT ATC TAC GGA
    1220
Leu Cys Tyr Leu Cys Glu Asn Ala Glu Arg Ser Ala Cys Ile Tyr Gly
              120             125             130

GGG GTA ACA AAT CAT GAA GGG AAT CAT TTA GAA ATT CCT AAA AAG ATA
    1268
Gly Val Thr Asn His Glu Gly Asn His Leu Glu Ile Pro Lys Lys Ile
              135             140             145

GTC GTT AAA GTA TCA ATC GAT GGT ATC CAA AGC CTA TCA TTT GAT ATT
    1316
Val Val Lys Val Ser Ile Asp Gly Ile Gln Ser Leu Ser Phe Asp Ile
            150             155             160

GAA ACA AAT AAA AAA ATG GTA ACT GCT CAA GAA TTA GAC TAT AAA GTT
    1364
Glu Thr Asn Lys Lys Met Val Thr Ala Gln Glu Leu Asp Tyr Lys Val
            165             170             175

AGA AAA TAT CTT ACA GAT AAT AAG CAA CTA TAT ACT AAT GGA CCT TCT
    1412
Arg Lys Tyr Leu Thr Asp Asn Lys Gln Leu Tyr Thr Asn Gly Pro Ser
180             185             190             195

AAA TAT GAA ACT GGA TAT ATA AAG TTC ATA CCT AAG AAT AAA GAA AGT
    1460
Lys Tyr Glu Thr Gly Tyr Ile Lys Phe Ile Pro Lys Asn Lys Glu Ser
              200             205             210

TTT TGG TTT GAT TTT TTC CCT GAA CCA GAA TTT ACT CAA TCT AAA TAT
    1508
Phe Trp Phe Asp Phe Phe Pro Glu Pro Glu Phe Thr Gln Ser Lys Tyr
              215             220             225
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CTT ATG ATA TAT AAA GAT AAT GAA ACG CTT GAC TCA AAC ACA AGC CAA
   1556
Leu Met Ile Tyr Lys Asp Asn Glu Thr Leu Asp Ser Asn Thr Ser Gln
        230                      235                 240

ATT GAA GTC TAC CTA ACA ACC AAG TAA CTTTTTGCTT TTGGCAACCT
   1603
Ile Glu Val Tyr Leu Thr Thr Lys  *
   245                 250

TACCTACTGC TGGATTTAGA AATTTTATTG CAATTCTTTT ATTAATGTAA AAACCGCTCA
   1663

TTTGATGAGC GGTTTGTCT  TATCTAAAGG AGCTTTACCT CCTAATGCTG CAAAATTTTA
   1723

AATGTTGGAT TTTTGTATTT GTCTATTGTA TTTGATGGGT AATCCCATTT TTCGACAGAC
   1783

ATCGTCGTGC CACCTCTAAC ACCAAAATCA TAGACAGGAG CTTGTAGCTT AGCAACTATT
   1843

TTATCGTC
   1851
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(2) INFORMATION FOR SEQ ID NO:13:

        (i) SEQUENCE CHARACTERISTICS:
             (A) LENGTH: 252 amino acids
             (B) TYPE: amino acid
             (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: protein

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

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Met Glu Asn Asn Lys Lys Val Leu Lys Lys Met Val Phe Phe Val Leu
  1               5              10                  15

Val Thr Phe Leu Gly Leu Thr Ile Ser Gln Glu Val Phe Ala Gln Gln
            20              25                  30

Asp Pro Asp Pro Ser Gln Leu His Arg Ser Ser Leu Val Lys Asn Leu
            35              40                  45

Gln Asn Ile Tyr Phe Leu Tyr Glu Gly Asp Pro Val Thr His Glu Asn
        50              55                  60

Val Lys Ser Val Asp Gln Leu Leu Ser His His Leu Ile Tyr Asn Val
  65              70                  75                  80

Ser Gly Pro Asn Tyr Asp Lys Leu Lys Thr Glu Leu Lys Asn Gln Glu
                85              90                  95

Met Ala Thr Leu Phe Lys Asp Lys Asn Val Asp Ile Tyr Gly Val Glu
            100             105                 110

Tyr Tyr His Leu Cys Tyr Leu Cys Glu Asn Ala Glu Arg Ser Ala Cys
        115             120                 125

Ile Tyr Gly Gly Val Thr Asn His Glu Gly Asn His Leu Glu Ile Pro
```

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          130                      135                      140
Lys Lys Ile Val Val Lys Val Ser Ile Asp Gly Ile Gln Ser Leu Ser
145                      150                      155                      160

Phe Asp Ile Glu Thr Asn Lys Lys Met Val Thr Ala Gln Glu Leu Asp
                    165                      170                      175

Tyr Lys Val Arg Lys Tyr Leu Thr Asp Asn Lys Gln Leu Tyr Thr Asn
                    180                      185                      190

Gly Pro Ser Lys Tyr Glu Thr Gly Tyr Ile Lys Phe Ile Pro Lys Asn
                    195                      200                      205

Lys Glu Ser Phe Trp Phe Asp Phe Phe Pro Glu Pro Glu Phe Thr Gln
          210                      215                      220

Ser Lys Tyr Leu Met Ile Tyr Lys Asp Asn Glu Thr Leu Asp Ser Asn
225                      230                      235                      240

Thr Ser Gln Ile Glu Val Tyr Leu Thr Thr Lys      *
                    245                      250
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